Non-Regenerative Benefits of Adult Bone Marrow Derived Stem Cells for Myocardial Protection

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A thesis presented by

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Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. I have designed and conducted all experiments together with the analysis of data. The following aspects of the thesis did require expert assistance and I worked in collaboration with the following:

1. Dr Garry Rucklidge, Department of Proteomics (University of Aberdeen), for proteomic gel electrophoresis and spot analysis
2. Dr Gary Duncan, Department of Proteomics (University of Aberdeen), for protein mass spectra analyses.
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I dedicate this thesis to my beloved and honourable Baba Jee.

I could never thank you enough for the unrelenting support and encouragement throughout my life, particularly during my academic progress.

I will never forget the words you first said to me:

“Never lose hope, prosperity is always delayed”.

Publications

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Abstracts:

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1) Sivarajah A, Collino M, **Yasin M**, Benetti E, Gallicchio M, Mazzon E, Cuzzocrea S, Fantozzi R, Thiemermann C. (2009). Anti-apoptotic and anti-inflammatory effects of hydrogen sulphide in a rat model of regional myocardial I/R. Shock 31(3):267-74

Abstract

Ischaemic heart disease is the most common cause of mortality in the western hemisphere and it is rapidly becoming the leading cause of death globally. Moreover, therapeutic interventions by cardiologists and cardiac surgeons frequently subject the heart to acute I/R injury, which in itself can cause mortality.

Recent investigations of adult stem cells have primarily focused on their regenerative potential for chronic ischaemic heart disease. In this thesis, I have investigated the hypothesis that adult bone marrow derived stem cells are cardioprotective in acute regional myocardial I/R injury. In a rat model of left anterior descending coronary artery (LAD) reversible occlusion and reperfusion, I demonstrate that an intravenous bolus of adult bone marrow derived (1) bone marrow mononuclear (BMNNC) and (2) mesenchymal stem cells (MSC) upon reperfusion can attenuate infarct size. This effect is comparable to ischaemic preconditioning (IPC), which is the gold standard for cardioprotection.

Next, I demonstrated the mechanisms for adult stem cell cardioprotection are principally anti-apoptotic and depend upon stem cell secreted factors to (1) activate phosphatidylinositide 3-kinase (PI3)/Akt cell survival kinase-signaling pathway (2) inhibit glycogen synthase kinase-3β (3) inhibit p38MAPK (4) inhibit nuclear translocation of p65NF-κB.

Proteomic analysis of myocardium subjected to I/R and treated with either BMMNC or BMMNC derived supernatant (BMS) upon reperfusion demonstrated higher expression of a whole host of pro-survival proteins. These were notably (1) 14-3-3-ε protein (2) anti-oxidant peroxiredoxin-6 (3) heat shock protein (HSP) αB-crystallin, HSP72, HSP tumour necrosis factor receptor-1 associated protein, and HSP ischaemia responsive protein-94 (4) glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (5) mitochondrial aconitase and mitochondrial voltage-dependent anion-selective channel protein-1.

Thereafter, I investigated the mobilization of endogenous bone marrow stem cells and trafficking to the ischaemic myocardium by stromal cell derived factor-1 (SDF-1) /chemokine, receptor type 4 (CXCR4) signaling. I demonstrate high up-regulated expression of CXCR4 and CD26 in BMMNC following IPC, which might have a role in IPC-mediated cardioprotection. Finally, and in concordance with this finding I demonstrate that both IPC and an exogenous MSC bolus upon reperfusion can synergize to abolish acute myocardial I/R injury.

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Abbreviations

Biological

AMI	acute myocardial infarction
BM	whole bone marrow
BMMNC	bone marrow mononuclear cells
BMS	BMMNC supernatant
CXCR4	chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle Media
DNA	deoxyribonucleic acid
ECM	extracellular matrix
ESC	embryonic stem cells
FBS	foetal bovine serum
GCSF	granulocyte colony stimulating factor
HGF	hepatocyte growth factor
HIF-1	hypoxia inducible factor-1
HSP	heat shock protein
IGF	insulin-like growth factor
LAD	left anterior coronary artery
LV	left ventricle
MMP	metalloproteinase
α-MEM	alpha-Modified Eagles Medium
MSC	mesenchymal stem cells

IM	intramyocardial
IP	intraperitoneal
IV	intravenous
SDF-1	stromal cell derived factor-1
TIMP	tissue inhibitor of metalloproteinase
VEFG	vascular endothelial growth factor

Biochemical and chemical

ATP	adenosine triphosphate
Ca^{2+}	calcium ion
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
FLICA	Fluorescent Labeled Inhibitor of Caspases
EDTA	ethylenediaminetetraacetic acid
H^+	proton
HBSS	Hank's balanced salt solution
H_2O_2	hydrogen peroxide
IL	interleukin
IPC	ischaemic preconditioning
I/R	ischaemia and reperfusion
K^+	potassium ion
K_{ATP}	ATP-sensitive potassium channel
MAPK	mitogen-activated protein kinases

MPTP	mitochondrial permeability transitional pore
Na^+	sodium ion
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	*p*-nitroblue tetrazolium
NCX	sodium/calcium exchanger
NF-κB	nuclear factor-kappa B
NHE-1	sodium/hydrogen ion exchanger-1
Na^+/K^+/ATPase	sodium/potassium ion exchanger
NO	nitric oxide
$O_2^{-\cdot}$	superoxide anion
$OH^{\cdot}$	hydroxyl radical
ONOO-	peroxynitrite
PBS	phosphate-buffered saline
PIC	protease inhibitor cocktail
PKC	protein kinase C
PTK	protein tryrosine kinase
PVDF	polyvinylidene fluoride
ROS	reactive oxygen species
SDSPAGE	sodium dodecyl-polyacrylamide

Measurements and units

AAR	area at risk
bpm	beats per minute
°C	degrees Celsius

h	hour
HR	heart rate
kDa	kilo Dalton
MAP	mean arterial pressure
mg/kg	milligram per kilogram
mg/ml	milligram per millilitre
min	minute
ml/kg	millilitre per kilogram
µl	microlitre
µl	micrometre
mm Hg	millimetres of mercury
µM	micromolar
mM	millimolar
%	percentage
PRI	pressure rate index
w/v	weight per volume
v/v	volume per volume

Miscellaneous terms

ANOVA	analysis of variance
2-D	2-dimensional
2-DE	2-dimensional gel electrophoresis
e.g.	for example

FACS	fluorescence activated cell sorter
FSC	forward scatter count
FITC	fluorescein isothiocyanate conjugate
g	acceleration due to gravity
i.e.	that is
n	number of experiments or animals
rpm	revolutions per min
SSC	side scatter count
SEM	standard error of the mean

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Chapter 1

General Introduction

1.1 Ischaemic Heart Disease

The heart is a muscular pump that sustains an adequate perfusion of blood to itself, the brain, and all other corporeal tissues. The heart exerts its function until death of the organism with the same or lesser number of cells than those present from birth (Chien 2004). Heart muscle, or myocardium, may lose a significant number of functional cells due to ischaemia. Ischaemia is a state of blood deprivation with a consequent denial of oxygen and other metabolic substrates. Myocardial ischaemia leads to cellular death, myocardial dysfunction and eventually death of the organism. Ischaemic heart disease is the leading cause of death in the West (Rosamond *et al.,* 2007). With the rapid economic development of the east, ischaemic heart disease is set to become the leading cause of death globally by 2020 (He *et al.,* 2005). Each year, 19 million people worldwide experience the morbidity and mortality of an ischaemic myocardial episode (Naghavi *et al.,* 2003). Ischaemic heart disease is also an economic burden, for example in the United Kingdom; the National Health Service spends £1.7 billion annually on ischaemic heart disease patients. United States, in comparison, spend $186 billion annually on ischaemic heart disease (Cohn *et al.,* 1997).

The loss of human life and the costs to the global economy continues to drive the tremendous research effort to discover novel strategies to protect against ischaemic heart disease.

1.1.1 Pathophysiology of Myocardial Ischaemia

The myocardium is a syncytium of cells, or cardiomyocytes, which function in electro-mechanical synchrony and form 75% of the contractile mass of the heart (Brilla *et al.,* 1991; Zak 1974). This syncytium of cardiomyocytes are maintained in an extracellular matrix (ECM), which is necessary to maintain the functional integrity and consists of an intricate network of type 1 and type 3 collagenous fibres produced by numerous myofibroblasts (Zak 1974; Weber 1997). Around 60% of each cardiomyocyte consists of contractile proteins invested with a dense array of mitochondria occupying approximately 20% of the cardiomyocyte volume. This high ratio of myofibrils to mitochondria are necessary to generate the high levels of intra-cardiomyocyte adenosine 3′, 5′-triphosphate (ATP) by oxidative phosphorylation (Schaper *et al.,* 1985). Thus in comparison to other bodily organs, myocardial oxygen consumption is the highest per mass per minute; i.e. 10ml oxygen per minute per 100mg of myocardium. Consequently, more than 75% of the oxygen delivered to the heart is subject to immediate extraction from the myocardial coronary blood flow. This limited physiological reserve mandates significant increases in coronary blood flow to ensure a balance in oxygen demand and supply is met especially during states of increased myocardial contractility (Camici *et al.,*

1996; Feigl 1983; Klocke 1976). It therefore also follows that the myocardium poorly tolerates luminal coronary arterial stenoses that limit perfusion and subject the myocardium to profound hypoxia even at rest. Such coronary artery perfusion defects are the principal cause of myocardial ischaemia and associated myocardial dysfunction or mortality.

During the pathophysiological state of myocardial ischaemia, glucose becomes the primary substrate for anaerobic production of ATP, but this glycolytic process has a significantly lower yield of ATP than that produced by fatty acid oxidation (Camici *et al.,* 1996; Oliver *et al.,* 1994; Rouslin *et al.,* 1990). Persistent glycolysis leads to an accumulation of pyruvate and nicotinamide adenine dinucleotide, which inhibits pyruvate dehydrogenase activity and prevents pyruvate entry into the mitochondria (Salem *et al.,* 2002). Accumulating pyruvate conversion to lactic acid leads to profound acidosis (Zemgulis *et al.,* 2001). Inevitably, glycolysis cannot generate sufficient ATP for myofibril contractility and maintenance of the important ion exchange mechanisms such as sodium/potassium ion pump activity (Na^+/K^+/ATPase), which is necessary for normal myocyte membrane polarity. Thus, continued dependence upon anaerobic metabolism rapidly leads to myocardial hypokinesis and accumulation of intra-myocyte Na^+. Intra-cardiomyocyte acidosis causes further Na^+ accumulation by increased sodium/hydrogen ion exchanger-1 (NHE-1) activity (Karmazyn *et al.,* 1999). Overload of Na^+ switches the sodium/calcium exchanger (NCX) into reverse mode to lower intra-myocyte

Na^+ but at the expense of increased intracellular Ca^{2+}. The combined Na^+ and Ca^{2+} overload leads to osmotic cellular swelling, loss in cell membrane integrity, and necrotic cell death (Murphy *et al.,* 2008; Pedersen 2006). Ischaemic cell death is primarily by necrosis, as it proceeds in the absence of ATP, and contrasts cell death by dependence on ATP i.e. apoptosis. Consequently, 70% of the myocardium will undergo necrosis following 3 h of continuous ischaemia. Termination of myocardial ischaemia by restoration of an adequate coronary blood flow is termed *reperfusion* and this is an essential first step in limiting injury to the ischaemic myocardium.

1.1.2 Pathophysiology of Myocardial Reperfusion

Reperfusion of an ischaemic myocardium limits necrotic injury and attenuates acute myocardial infarction (AMI) by 40% (Reimer *et al.,* 1977). Reperfusion, however, can propagate tissue injury beyond that sustained by ischaemia alone (Jennings *et al.,* 1960; Braunwald *et al.,* 1985). This paradoxical effect of reperfusion following ischaemia is widely termed reperfusion injury. At the onset of reperfusion, there is a rapid production of reactive oxygen species (ROS), correction of intra-cellular acidaemia, but this is at the expense of further Na^+ overload, Ca^{2+} overload, and consequential depression of myocardial contractility or stunning. Thus, during prolonged ischaemia over activity of NHE-1 leads to Na^+ overload and secondarily to Ca^{2+} overload due to reverse activity of the NCX. ROS can further increase NHE-1 activity by H_2O_2 mediated phosphorylation by mitogen activated protein kinases (MAPK), which include p38, c-jun, NH2-

terminal kinase (JNK) and extracellular signal-regulated kinases 1/2 (Sabri *et al.,* 1998; Wei *et al.,* 2001; (Snabaitis *et al.,* 2002); Moor *et al.,* 2001). NHE-1 activity is at its peak during the initial period of reperfusion and results in a greatly increased intracellular Na^+ concentration, which affects the direction of NCX. NCX activity is important for the removal of intracellular Ca^{2+} from the cardiomyocyte, but NCX is sensitive to sub-sarcolemmal Na^+. Thus, the increased intracellular Na^+ causes a reverse mode NCX action leading to Ca^{2+} influx into the cardiomyocyte. ROS can also directly increase NCX activity as shown by exposure of cardiomyocytes to H_2O_2 (Goldhaber 1996). Accumulating cytosolic Ca^{2+} leads to increased mitochondrial Ca^{2+} and causes mitochondrial swelling with eventual mitochondrial outer membrane disruption (Shen *et al.,* 1972). Loss of mitochondrial outer membrane integrity and leakage of mitochondrial pro-apoptotic factors triggers apoptotic cell death.

An important combined effect of ROS and mitochondrial calcium overload during reperfusion leads to the opening of a non-specific inner mitochondrial membrane pore called the mitochondrial permeability transitional pore (MPTP). Opening of MPTP is detectable 2 to 3 minutes after the onset of myocardial reperfusion but not during ischaemia (Halestrap *et al.,* 2004). MPTP opening leads to uncontrolled proton entry into the mitochondrial matrix and loss of the inner mitochondrial membrane potential or proton motive force, which is essential for oxidative phosphorylation. This not only decelerates mitochondrial ATP production

but also accelerates hydrolysis of all cellular ATP to cause a rapid decline in the total cellular ATP levels (Halestrap *et al.,* 2004). The primary trigger for MPTP opening is increase in matrix calcium (Crompton *et al.,* 1987), and sensitivity to opening is further increased by mitochondrial ROS, phosphate, and mitochodrial membrane depolarization (Halestrap 2009). The maintenance of MPTP closure is by the normally high levels of ATP, ADP, Mg^{2+}, and H^{+} within the mitochondrial matrix. The molecular components of MPTP have not been entirely characterised, however, the discovery of Cyclophilin D (CypD) as a facilitator of MPTP opening in the presence of calcium and phosphate was a milestone in the understanding of the MPTP mechanism (Crompton *et al.,* 1988). States of calcium overload lead to increased CypD binding to the mitochondrial phosphate carrier (PiC) to enable MPTP activation. This facilitating role of CypD during calcium overload can be desensitized by the sub-micromolar concentrations of the immunosuppressant Cyclosporin A (Leung *et al.,* 2008b). The key MPTP pore forming protein(s) remain elusive to date. Adenine nucleotide translocase (ANT) may be an MPTP pore forming protein as it binds to ATP or its deoxyribose analogues to reduce the calcium sensitivity of MPTP opening (Halestrap *et al.,* 1997). Oxidative stress or the "c" conformation caused by carboxyatractyloside prevents the binding of ANT to adenine nucleotides (Halestrap *et al.,* 1997). Data that is more recent however suggests genetic ablation of ANT significantly reduces MPTP opening sensitivity to calcium but does not abolish it (Kokoszka *et al.,* 2004 4551).

This suggests that ANT may not be the essential protein for MPTP pore formation. Other data points to PiC as being a possible MPTP pore forming protein (Leung *et al.,* 2008a). There are however, other proteins that might have a role in the formation of MPTP pore and of which a 32kDa protein identified as voltage dependent anion channel isoform-1 (VDAC-1) is supported by the strongest evidence (Cesura *et al.,* 2003; Crompton *et al.,* 1998).

Reperfusion of the ischaemic myocardium results in significant depression in myocardial function (Braunwald *et al.,* 1982; Heyndrickx *et al.,* 1975). During reperfusion the myocardial myofilaments have decreased sensitivity to Ca^{2+}. The exact mechanism for this is not yet resolved. Overload of Ca^{2+} may cause proteolysis of the myofilament regulatory proteins such as Troponin I (Gao *et al.,* 1996; Mcdonough *et al.,* 1999; Van Eyk *et al.,* 1998). Other studies argue against this suggested mechanism (Feng *et al.,* 2001; Kim *et al.,* 2001; Thomas *et al.,* 1999). Overload of Ca^{2+} and MAPK activation, particularly overexpression of JNK and p38 MAPK, can also lead to myofibrillar disassembly.

Reperfusion of the ischaemic myocardium is associated with an exponential production of ROS, which also contributes directly to the persistent myocardial dysfunction (Bolli *et al.,* 1988). ROS may directly decrease contractility by decreased myofibrillar ATPase, decreasing creatine kinase activity, stimulating myosin phosphatase, or by metabolic depletion

of ATP (Hein *et al.,* 1995; Shattock 1997). ROS are bio-reactive free radicals including superoxide anion radical (O_2^{-}.), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), lipid peroxide (ROO·), and peroxynitrite ($ONOO^-$). The reactions that generate ROS have been summarised in table 1.1.2.1. Electron paramagnetic spin resonance spectroscopy has demonstrated that although ROS are generated during ischaemia, the greatest increase in ROS generation occurs during the initial moments of reperfusion (Bolli *et al.,* 1988; Zweier *et al.,* 1987). This explosive burst of ROS occurs within minutes of reperfusion and mandates presence of oxygen (Zweier *et al.,* 1987). In the ischaemic and reperfusing heart, ROS generation is by cardiomyocytes, endothelium and neutrophils. The mitochondria are the major intracellular source of ROS, particularly complexes 1 and 3 of the electron transport chain (Turrens 2003). ROS have profound deleterious effects on the myocardium, which include peroxidation of lipids and proteins. Thus, ROS can interfere with the normal functions of various intracellular targets including second messenger pathways, L-type Ca^{2+} channels, potassium channels, ion transporters, and contractile proteins (Park *et al.,* 1999). Cardiomyocytes' exposure to O_2^{-}. and H_2O_2 has been shown to inhibit active Na^+ and Ca^{2+} pumps and at the same time activate NCX (Goldhaber *et al.,* 1994). ROS also reduce myofibrillar ATPase activity, which can attenuate the responsiveness to Ca^{2+} and contributes to myocardial stunning(Ventura *et al.,* 1985). Under physiological conditions, ROS levels are low due to the mitochondrial antioxidant defence

mechanisms, which include enzymes such as superoxide dismutase (SOD), glutathione peroxidase, and glutathione reductase. Mitochondrial non-enzymatic anti-oxidants include glutathione and α-tocopherol and coenzyme Q. Antioxidants act by scavenging oxidative species and their precursors. However, during I/R the balance between oxidants and antioxidants is perturbed, resulting in oxidative stress. Thus, amelioration of ROS by the use of scavengers of free radicals such as catalase and superoxide dismutase can reduce reperfusion injury (Zweier *et al.,* 1987). In concordance with this, the Cambridge Heart Antioxidant Study (CHAOS) trial demonstrated a decreased incidence of cardiovascular events in patients with coronary disease following treatment with vitamin E (Stephens *et al.,* 1996).

During I/R excessive ROS exerts many deleterious effects on myocardial tissue by reacting with membrane lipids, proteins and nucleic acids to alter their physiological function. ROS have been demonstrated to induce lipid peroxidation in reperfused hearts (Ambrosio *et al.,* 1991; Paradies *et al.,* 2004; Paradies *et al.,* 1999). This process results in the fragmentation of fatty acids and in the formation of lipid peroxidation products, the products of which are cytotoxic. During the massive release of ROS upon reperfusion membrane ion channel proteins, that play a key role in the homeostasis of cardiomyocytes, alter their permeability to K^+, Ca^{2+} and to Na^+. The activities of ion pumps such as Na^+/K^+/ATPase and sarcolemmal Ca^{2+}/ATPase decreases after the exposure to oxidative stress. The ischaemic myocyte has an accumulation of intracellular Ca^{2+} due to

increased activity of reverse mode NCX and there is a further increase in cytosolic Ca^{2+} upon reperfusion(Meissner *et al.,* 1995). Additonal contributors for Ca^{2+} overload include decreased sarcoplasmic reticulum reuptake, decreased Ca^{2+} extrusion, increased entry from voltage-sensitive Ca^{2+} channels. The Ca^{2+} overload does not increase myocardial contrctility as the oxidative modification of contractile proteins causes myocardial stunning and haemodynamic failure.

In addition to the cell damaging effects of ROS, reperfusion of the previously ischaemic heart is also associated with an intense inflammatory reaction and the recruitment of leukocytes into the reperfusing myocardium (Engler *et al.,* 1986). Engler *et al.,*, have shown that compared to regional ichaemia alone, reperfusion greatly enchanced infiltration of neutrophils into the reperfusing myocardium. Neutrophil migration from the circulation into the myocardium is via a complex sequence of molecular steps involving selectins and integrins, which are a group of cellular adhesion molecules that mediate leukocyte adhesion and rolling to the endothelium. Neutrophils extravasate by leucocyte integrin (CD11/CD18) interactions with endothelial intercellular adhesion molecule-1 (ICAM-1) so permitting neutrophil adhesion to the endothelium and subsequent transmigration (Entman *et al.,* 1991). Complement fragments such as C5a(Mueller *et al.,* 2013) and cytokines such as tumour necrosis factor alpha (TNF-α), interleukin (IL)-1, IL-6 or IL-8 act as chemoattractant factors that stimulate neutrophil mediated events following I/R of the heart. Neutrophil

stimulation with chemotactic factors (e.g. C5a, IL-8, PAF, f-Met-Leu-Phe) and endothelial stimulation by inflammatory cytokines (e.g. IL-1, TNFα, endotoxin) significantly augments neutrophil transmigration (Smith *et al.,* 1989; Smith *et al.,* 1988). Transmigrated neutrophils then bind to cardiomyocytes expressing ICAM-1 and thereby inject a burst of NADPH oxidase generated ROS and proteolytic enzymes into ischaemic and reperfusing myocytes (Entman *et al.,* 1992). These neutrophil mediated cytotoxic effects are necessary to remove non-viable cardiomyoctes to facilitate repair and remodelling post I/R (Richard *et al.,* 1995; Roberts *et al.,* 1976). Subsequent to the acute inflammatory repsonse, adaptive immune cells (i.e. monocytes and lymphocytes) are recruited to the reperfusing myocardium to enable repair and adverse remodelling (Yan *et al.,* 2013).

Table 1.1.2.1: ROS generating reactions

Reduction of O_2	$O_2 + e^- \rightarrow \cdot O_2^-$ $\cdot O_2 + e^- + 2H+ \rightarrow H_2O_2$ $\cdot O_2^- + H_2O_2 + H+ \rightarrow O2 + H_2O + \cdot OH$ $\cdot OH + e^- + H+ \rightarrow H_2O$
Iron-related	$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH-$ (Fenton reaction) $Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2$ $\cdot O_2^- + H_2O_2 \rightarrow O_2 + \cdot OH + OH-$ (Haber-Weiss reaction)
Lipid peroxidation	Lipid-H + $\cdot OH \rightarrow H_2O$ + Lipid· Lipid· + $O_2 \rightarrow$ Lipid OO· Lipid OO· + Lipid-H → Lipid-OOH + Lipid· Lipid· + Lipid· → Lipid-Lipid
Xanthine-oxidase	ATP→adenosine→xanthine Xanthine + H_2O +$2O_2$→uric acid + $2\cdot O_2^-$ +2 H^+
NADPH oxidase	NADPH + $2O_2$→ $NAD(P)^+ + H^+ + 2\cdot O_2^-$
Peroxynitrite	$\cdot NO + \cdot O_2^- \rightarrow ONOO^-$

1.1.3 Protecting the Myocardium from Ischaemia Reperfusion Injury

Protecting the myocardium from ischaemia requires emergent restoration of myocardial blood flow and the degree of injury due to ischaemia and subsequent reperfusion is critically dependent upon the duration of ischaemia. The likelihood of developing cardiac failure and the subsequent prognosis has a strong correlation with the infarct size (Page *et al.,* 1971; Sobel *et al.,* 1972). Thus, attenuation of ischaemia reperfusion (I/R) injury is possible by keeping the ischaemia time to a minimum and also by reducing the myocardial demand for oxygen during ischaemia. Pharmacological interventions that reduce myocardial oxygen demand include β_1-adrenoreceptor-blockers, diuretics and vasodilators. In those patients with acute coronary artery occlusion, the ischaemia time is kept to a minimum by efficient emergency services keeping the time from onset of chest pain to thrombolytic therapy or PCI to less than 90 min. Cardiac surgical procedures can reduce the myocardial oxygen consumption by 90% using cardioplegic coronary perfusion and topical hypothermia to further reduce oxygen consumption by 7% per °C fall in temperature (Bigelow *et al.,* 1950; Braimbridge *et al.,* 1977; Buckberg 1979; Melrose *et al.,* 1955; Teoh *et al.,* 1986). Reducing myocardial oxygen consumption by cardioplegia and hypothermia enables the heart to tolerate significantly long periods of global ischaemia during open-heart surgery. These techniques, however, cannot completely abolish myocardial oxygen consumption and

very long ischaemia times frequently lead to profound myocardial injury, multi-organ failure syndrome, and death.

An innate cardioprotective mechanism that can reliably minimise the infarct size due to I/R is called ischaemic preconditioning (IPC) and this reported to be the most powerful discovered to date. Myocardial IPC follows brief periods of ischaemia of approximately 3-5min duration, which can then prevent the significant injury caused by a subsequent prolonged period of ischaemia(Jennings *et al.,* 1995; Yellon *et al.,* 1992). IPC was first described in a canine model whereby four 5 min cycles of regional myocardial ischaemia by left anterior descending coronary artery (LAD) reversible occlusion and reperfusion. A subsequent 40 min LAD occlusion was associated with significant reductions in infarct size, when compared with controls (Murry *et al.,* 1986). This powerful endogenous cardioprotection has been also been demonstrated in the rat heart (Liu *et al.,* 1992b; Liu *et al.,* 1992a), the rabbit heart (Thornton *et al.,* 1990), the pig heart(Schott *et al.,* 1990), and in the human heart (Yellon *et al.,* 1993). The mechanism for IPC remains elusive despite the 25 years or so since its discovery. Current evidence suggests that IPC activates a complex signaling cascade including G-protein coupled receptors(Schultz *et al.,* 1998), Janus activated kinase/signal transducers and activators of transcription (JAK/STAT) (Bolli *et al.,* 2003), Src tyrosine kinases (Ping *et al.,* 1999), phosphatidylinositol-3 kinase (Tong *et al.,* 2000), ROS (Hausenloy *et al.,* 2007), protein kinase C-epsilon (Inagaki *et al.,* 2006), glycogen synthase

kinase-β inhibition (Tong *et al.,* 2002), and inhibition of the MPTP (Juhaszova *et al.,* 2004). IPC as a feasible therapeutic option is only possible when I/R is anticipated e.g. cardiopulmonary bypass surgery (Jenkins *et al.,* 1997; Yellon *et al.,* 1993), and percutaneous transluminal coronary angioplasty (Deutsch *et al.,* 1990). But most AMI patients present hours after the onset of acute myocardial ischaemia, thus precluding IPC therapy.

The search for additional myocardial protection strategies has led to the publication of over 14000 experimental myocardial protective strategies since 1970. Despite these impressive research efforts, translation of these putative myocardial protective strategies into actual clinical practice remains elusive (Bolli *et al.,* 2004). Further, a significant number of patients experience acute myocardial ischaemia out of hospital (Lloyd-Jones *et al.,* 2010) and most of these patients either do not survive the onset of myocardial ischaemia or they present to hospital services many hours or even days, following the onset of myocardial ischaemia (Zheng *et al.,* 2001). Thus, novel strategies to protect the heart against reperfusion injury and regenerate a myocardial infarct remain highly sought after. Most importantly, there are no current therapies for AMI that target the pathogensis of myocardial injury (e.g. ROS production during I/R) or facilitate the regeneration of damaged myocardium.

1.2 Stem Cell Therapy Post Acute Myocardial Infarction

Following AMI, cells that originate from within the viable myocardium and cells from the circulation invade the infarct zone (Bing 2001; Cleutjens 1996; Fishbein *et al.,* 1978; Frangogiannis *et al.,* 2002). Inflammatory macrophages remove dead cells and fibroblasts proliferate to replace the vacated space with dense collagenous scar tissue. Scarring is associated with modulation of the ECM and contributes to aneurysmal ventricular dilation, diastolic dysfunction, and systolic dysfunction (Jugdutt 2003). Adverse remodeling has long been thought to be an inevitable and irreversible consequence of myocardial injury. This has, however, recently been challenged by reports that suggest resident cardiac stem cells differentiate into cardiomyocytes to provide regenerative potential (Anversa *et al.,* 2006; Beltrami *et al.,* 2003; Nadal-Ginard *et al.,* 2003; Urbanek *et al.,* 2005). Nevertheless, in reality the endogenous cardiac stem cells fail to regenerate a myocardial infarct. The reasons for this are not entirely clear and this may simply be due to a significant loss of cardiac stem cells within the infarct zone. Hence, an exogenous stem cell resource might redress the balance and lead to myocardial repair or regeneration.

1.2.1 Exogenous Stem Cells

Stem cells are unique in that they replicate for self-maintenance and provide daughter cells that undergo differentiation for specialized function

(Till *et al.,* 1961). Stem cells are either embryonic or adult. Embryonic stem cells (ESC) constitute the embryonic blastocyst and generate all three germ cell layers the ectoderm, mesoderm and endoderm. ESCs are termed totipotential because of their potential to generate all tissue cell types (Amit *et al.,* 2000; Thomson *et al.,* 1998a; Thomson *et al.,* 1998b). Thus, ESCs will differentiate into functional cardiomyocytes (He *et al.,* 2003; Kehat *et al.,* 2001; Takahashi *et al.,* 2003; Ventura *et al.,* 2003; Wobus *et al.,* 1997; Xu *et al.,* 2002; Xue *et al.,* 2005). In contrast to ESCs, adult stem cells are postnatal and restricted in their lineage of differentiation, e.g. haematopoietic stem cells will only reconstitute blood cells. More recently, this lineage restriction has been challenged and the concept of adult stem cell differentiation has evolved to include transdifferentiation; bone marrow derived haematopoietic stem cell can differentiate into cells of the brain, liver and heart, (Blau *et al.,* 2001; Korbling *et al.,* 2003). Which exogenous stem cell type is most appropriate for regenerating the infarcted myocardium? This is still open to debate.

1.2.1.1 Embryonic Stem Cells and Cardiac Repair

Embryonic stem cells (ESCs) derive from the inner cell mass of the blastocyst, which is complete on day five following fertilization. Isolated human ESCs can proliferate indefinitely in the presence of leukaemia inhibitory factor or on a feeder layer of mitotically inactivated mouse embryonic fibroblasts to provide a stable ESC source (Lebkowski *et al.,* 2001). ESCs are the ideal cell type for stem cell therapy following

myocardial infarction, because they can easily proliferate in culture to provide the large numbers of cells necessary and more importantly they can be made to differentiate into functional cardiomyocytes both *in vitro* and *in vivo* (Kehat *et al.,* 2001; Min *et al.,* 2002). Despite the advantages of ESC, their clinical employment is restricted by a number of problems including ethical issues, potential for immune rejection, and the potential for intra-ventricular teratomas (Laflamme *et al.,* 2005).

1.2.1.2 Adult Stem Cells and Cardiac Repair

The bone marrow is the primary source for obtaining adult stem cells. The most important subpopulation of bone marrow cells used in cardiac stem cell therapy is the bone marrow mononuclear cell (BMMNC). BMMNC contain mesenchymal stem cells (MSC), haematopoietic stem cells (HSC), endothelial progenitor cells (EPC), natural killer cells, T-lymphocytes, and B-lymphocytes. Alternative sources of adult stem cells include adipose tissue, placental tissue, umbilical cord blood and peripheral blood.

The earliest report of an adult stem cell application for cardiac repair was by Orlic et al., involving the transplantation of haematopoietic stem cells (enriched for c-Kit+ Lin-) into the border zone of an ischaemia damaged mouse myocardium. The transplanted adult bone marrow derived stem cells (c-Kit+ Lin-) could differentiate into all the component cells required to produce *de novo* myocardium, which occupied 68% of the

infarct zone 9 days following injection (Orlic *et al.,* 2001). Interestingly, Jackson *et al.,* reported similar myocardial regenerative capacity when a side population of haematopoietic stem cells was transplanted into recipient bone marrow (Jackson *et al.,* 2001). These initial studies suggested that donor adult stem cells migrate in response to signaling from the injured heart and then differentiate into a myocardial cell lineage. Further, xenogeneic donor cells are also cardioprotective; human adult haematopoietic stem cells when injected into rat myocardial infarcts transdifferentiated into vascular endothelial cells, prevented cardiomyocyte apoptosis, and prevented adverse remodeling (Kocher *et al.,* 2001).

The initial exciting reports suggesting the regenerative benefits of adult bone marrow derived stem cells for myocardial protection have, however, been dampened by later reports suggesting that transplanted adult bone marrow derived stem cells could not modulate their lineage of differentiation for myocardial regeneration (Balsam *et al.,* 2004; Murry *et al.,* 2004). The regenerative benefits of adult stem cells were further questioned by reports that suggested donor adult bone marrow stem cell mechanism of protection might be by the secretion of paracrine factors, which could attenuate cardiomyocyte death, following myocardial infarction (Gnecchi *et al.,* 2006; Uemura *et al.,* 2006). Nevertheless, autologous unfractionated bone marrow mononuclear cells (BMMNC) derived from adult bone marrow have been investigated in clinical trials. After AMI, patients were revascularized by percutaneous coronary intervention and

randomized to receive a single dose of intra coronary BMMNC (Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006). Lunde *et al.,* reported no improvement in left ventricular ejection fraction (LVEF) at 6 months; Meyer *et al*., reported an initial improvement at 6 months, but no improvement in LVEF 18 months following intracoronary injection; Schachinger *et al*., reported a 5% improvement in LVEF at 4 months and lower mortality from major adverse cardiac events at 1 year. These clinical trials did not replicate the improvements in cardiac function suggested by the *in vivo* experimental data. These disparities may be due to differences in quality of autologous BMMNC, differences in the time of BMMNC injection, differences in the route of injection. In any case, these trials did not suggest a regenerative benefit of adult bone marrow stem cell therapy and possibly a paracrine effect might have been responsible for the improvement in LVEF suggested by Meyer *et al*., and Schachinger *et al*.

1.2.1.3 Mesenchymal stem cells

The adult stem cells contained in BMMNC are mainly haematopoietic stem cells. The BMMNC content of MSCs (< 0.01%) and endothelial progenitor cells (1-2%) is very small. Although a number of studies have disputed the regenerative benefits of bone marrow derived stem cells in cardiac repair, data from gender mismatched heart transplant and bone marrow transplant patients suggest that the adult bone marrow derived stem cells do contribute to cardiomyocyte turnover in the heart (Deb *et al.,* 2003; Quaini *et al.,* 2002). Moreover, Kawada *et al*. (2004), report that the

non-haematopoietic MSC sub-fraction can only differentiate into cardiomyocytes (Kawada *et al.,* 2004). This is concordant with the data from Pittenger *et al*. (1999), which suggests that MSCs from various sources differentiate into multiple non-haematopoietic lineages including adipose tissue, bone, cartilage and cardiomyocytes (Pittenger *et al.,* 1999). Further, bone marrow derived MSCs are able to differentiate into mature functional cardiomyocytes both *in vitro* and *in vivo* (Makino *et al.,* 1999; Tomita *et al.,* 1999).

As well as the regenerative benefits, MSC therapy also imparts non-regenerative benefits by paracrine-mediated improvement of cardiac function, cardiac perfusion and attenuation of apoptosis of the native cardiomyocytes (Dai *et al.,* 2005; Tang *et al.,* 2005). Initial clinical studies suggest bone marrow-derived MSCs improve cardiac function following AMI, but the mechanisms for these benefits are yet to be established (Chen *et al.,* 2004). A number of important issues currently prevent the use of MSC therapy becoming a realistic clinical option. These include optimization of the cell-culture expansion protocols, optimization of methods leading to cardiomyocyte differentiation, elimination of immunogenic factors in the culture media, identification of the optimal route of cell delivery, and identification of the optimal time of cell delivery.

1.2.1.4 Endothelial progenitor cells

Regeneration of *de novo* myocardium would necessitate migration of bone marrow derived endothelial progenitor cell (EPC) and their myocardial engraftment for neoangiogenesis. This process of vasculogenesis was suggested following the discovery of $CD34^+$ peripheral blood EPCs (Asahara *et al.,* 1997; Shi *et al.,* 1998). Haematopoietic stem cells share a common origin with EPCs by their expression of CD34 or CD133 and an endothelial marker such as vascular endothelial growth factor receptor 2. Kocher *et al*., investigated the regenerative benefits of freshly isolated $CD34^+$ EPCs adminstered IV in nude rats with myocardial ischaemia and found inhibition of cardiomyocyte apoptosis and preservation of left ventricular function (Kocher *et al.,* 2001). Similarly, IV injection of human EPCs expanded in culture migrated to the ischaemic nude rat myocardium to preserve ventricular function and attenuate fibrosis (Kawamoto *et al.,* 2001). The applicability of EPCs in routine clinical practice, however, is limited by the scarcity and functional impairment due to diabetes, aging, and hypercholesterolaemia (Hill *et al.,* 2003; Vasa *et al.,* 2001; Werner *et al.,* 2005). Alternative sources to assure quality and quantity of EPC therapy might be from allogeneic cord blood or culture expansion from ESCs (Levenberg *et al.,* 2002; Murohara *et al.,* 2000)

1.2.2 Modalities of Exogenous Stem Cell Delivery

The efficacy of stem cell therapy for repair of the myocardium is reliant upon their successful delivery to the site of injury. Stem cells are

delivered by three main ways: (1) IV (systemic or retrograde coronary) (2) intra-coronary (3) intra-myocardial. The ideal modality should be safe, cheap, easy, widely applicable, targeted and high retention of cells in the target area. Prior to stem cell delivery, a process of stem cell mobilization is necessary. Following AMI, physiological mechanisms mobilize endogenous bone marrow stem cell in humans to attempt cardiac repair (Shintani *et al.,* 2001). The MAGIC trial used granulocyte colony stimulating factor (G-CSF) to increase endogenous bone marrow stem cell mobilization prior to harvest and delivery (Kang *et al.,* 2004). Most exogenous stem cells delivery methods usually involve an invasive clinical procedure to harvest the cells prior to delivery, e.g. bone marrow aspiration.

The advantages of systemic IV stem cell delivery include simplicity, low cost, minimally invasive, and rapid donor stem cell migration down a chemokine gradient to target the ischaemic myocardium (Lew 2004). This cell delivery technique is critically reliant upon the chemokine signaling between the ischaemic myocardium and the peripheral circulation. Thus, optimal time of delivery by the IV route would be within 2 days of the AMI (Barbash *et al.,* 2003). A major drawback of systemic IV is the low retention of cells within the zone of injury (Aicher *et al.,* 2003; Barbash *et al.,* 2003). Thus, current literature suggests that the systemic IV route of stem cell delivery following AMI would not lead to significant regenerative benefit. There is however, no data to suggest that systemic IV injection of cells would not lead to a higher cell engraftment in the infarct zone if the

injection of cells were earlier than 24 h from the onset of ischaemia or reperfusion. Further, there is also no evidence that a particular threshold of cellular engraftment is necessary before any regenerative benefit is evident.

The retrograde coronary venous cell delivery is another percutaneous modality of stem cell delivery and has the advantages of increased contact time to enhance binding and myocardial concentration (Murad-Netto *et al.,* 2004; Von Degenfeld *et al.,* 2003). This technique of cell delivery is expensive and not widely available; it would require a cardiac catheter suite, X-ray imaging, and operator expertise. Further, the use of the coronary venous system for the delivery of cardioplegia during cardiac surgery suggests that the right ventricle cannot be adequately protected (Stirling *et al.,* 1989).

The intracoronary infusion also directly targets the myocardium and it is the most common method to clinically to deliver stem cells 4 days or more following an acute myocardial infarct (Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006). This route of cell delivery is widely available and uses the same technique for coronary angioplasty in the cardiac catheter suite. Thus, the stem cells target the culprit arterial territory and stopping the coronary blood flow for 2-4 minutes increases the homing time of cell injection. Stem cell homing is further increased by taking advantage of the up-regulated homing cytokines and adhesion molecules caused by the acute pathology. Despite this highly targeted approach to

deliver cells, the efficiency of cellular engraftment is still very poor following AMI (Hofmann *et al.,* 2005). Further, the injection of large cells such as MSCs could occlude the coronary microcirculation. Vulliet *et al*., report the occurrence of micro infarctions when healthy dogs received MSC via the intracoronary route (Vulliet *et al.,* 2004). Furthermore, the evidence base that suggests that intracoronary cell delivery is better than other routes for safety and efficacy is by no means strong. In the presence of extensive/occlusive coronary artery disease and numerous previous coronary interventions, this approach limits cell delivery.

Intramyocardial cell delivery is the preferred route of cell delivery in patients with chronic total occlusion or in permanent coronary ligation experimental models. It would also be logical to deliver larger cells such as skeletal myoblasts and MSCs intramuscularly to prevent microvascular plugging and micro infarctions following cell delivery (Vulliet *et al.,* 2004). Intramyocardial cell delivery can be via transepicardial, transendocardial, or transcoronary sinus. The transepicardial injection of the myocardium is the most invasive technique and requires a median sternotomy. Hence, this route is for patients undergoing surgical cardiac procedures, e.g. coronary artery bypass grafting. Despite the invasiveness and the associated morbidity, it does have a proven safety record (Orlic *et al.,* 2001; Silva *et al.,* 2005; Stamm *et al.,* 2003). The surgeon has to make a prior visual inspection of the myocardium to ensure optimal targeting of the cell injection. However, this technique will not allow inspection of the inter-

ventricular septum without either a right or left ventriculotomy. On the other hand, transendocardial delivery by percutaneous transfemoral insertion of the injection catheter avoids the morbidity of a median sternotomy. A more accurate targeting of cell delivery is possible by creating electromechanical maps of the endocardium to identify viable and non-viable myocardium (Sarmento-Leite *et al.,* 2003). The safety profile of this mode of cell delivery is excellent in both humans and animals (Fuchs *et al.,* 2001; Perin *et al.,* 2003). Transendocardial stem cell delivery in the AMI setting is yet to be fully assessed. Trancoronary sinus injection requires the placement of the injection catheter into the coronary sinus via a percutaneous transfemoral approach. This technique relies on intravascular ultrasound guided cell delivery and it has been shown to be feasible and safe (Thompson *et al.,* 2003). It is however limited because of the need for a high level of operator expertise and the challenges of negotiating coronary sinus tortuosity. The delivery of cells by intramuscular injection causes local injury and possibly an inflammatory reaction, which might jeopardize the injected cells and diminish their engraftment. Unlike the intravascular delivery modalities, the intramuscular injection does not lead to a widespread diffuse engraftment of cells, which instead form islets or cell clusters within the recipient myocardium (Leobon *et al.,* 2003; Reinecke *et al.,* 2002). If these injected cell clusters were to form *de novo* myocardium, they may not form electromechanical connectivity with the main syncytium, thus have no functional contribution, and may become foci of arrhythmias.

1.2.3 Stem Cell Homing Post AMI

Following acute myocardial injury, endogenous bone marrow stem cells mobilize and home into the infarct zone to attempt cardiac repair (Shintani *et al.,* 2001). The stem cell homing to the myocardium is by signaling between stromal cell derived factor-1 (SDF-1) and its cognate receptor chemokine receptor type 4 (CXCR4). Endothelial and stromal cellular lining of the bone marrow stem cell niche constitutively expresses SDF-1, which interacts with its cognate receptor CXCR4 expressed on the stem cells (Dar *et al.,* 2005; Imai *et al.,* 1999; Ponomaryov *et al.,* 2000). During homeostasis, endogenous bone marrow stem cells continually and variably egress into the blood stream in a circadian cyclical manner. These circadian changes in the levels of circulating bone marrow stem cells is under sympathetic control via noradrenaline binding to beta3-adrenoceptors on bone marrow stromal cells to decrease expression of SDF-1 and reduced CXCR4 signaling (Mendez-Ferrer *et al.,* 2008). These daily changes in bone marrow stem cell mobilisation are, however, slow and peak 5 h following exposure to light (Mendez-Ferrer *et al.,* 2008). In stress or pathological states, e.g. following AMI there is an elevation of SDF-1 in the heart, which intravasates to create a chemokine or SDF-1 gradient between the bone marrow stem cell niche and site of the myocardial infarction for trafficking of mobilised stem cells (Askari *et al.,* 2003; Ceradini *et al.,* 2004; Jiang *et al.,* 2002; Wojakowski *et al.,* 2004). Taking into account that SDF-1 is highly expressed by the bone marrow stem cell niche (Sugiyama *et al.,*

2006), a rapid and massive intravasation of bone marrow stem cells would require a commensurate reduction in SDF-1 activity within the bone marrow niche. That the endogenous bone marrow stem cell mobilisation is inadequate, exogenous cell delivery might be most beneficial if delivered before the onset of ischaemia or soon after the onset of reperfusion. Thus far, clinical trials delivered bone marrow derived stem cells more than 4 days following an AMI (Chen *et al.,* 2004; Janssens *et al.,* 2006; Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006).

1.2.4 Regenerative and Non-regenerative Benefits of Adult Stem Cell Therapy

The regenerative benefits of the injected stem cells are dependent upon the engraftment number, within an ischaemic or reperfusing myocardium. The engraftment number will in turn determine the number of cells that undergo differentiation and the lineage of differentiation will determine the functional benefit. Moreover, in the heart the appropriately differentiated cells must also achieve electromechanical connectivity with the remaining syncytium to improve cardiac function. Thus far, the engraftment number of donor cells is very low. Most cells are lost immediately after injection, regardless of donor cell type or the modality of cell injection (Hou *et al.,* 2005). Moreover, there is a high rate of loss of those cells that achieve initial engraftment (Muller-Ehmsen *et al.,* 2006). This low rate of myocardial engraftment and subsequent retention is poorly

understood and if it remains so, the regenerative benefits of stem cell therapy may remain elusive.

The differentiation of engrafted adult stem cells into the component cells necessary for myocardial regeneration remains controversial. The differentiation of MSCs into cardiomyocytes has been demonstrated *in vitro* by the application of 5-azacytidine (Makino *et al.,* 1999). Further, co-cultures of MSCs with cardiomyocytes have suggested differentiation is possible together with electromechanical integration (Fukuhara *et al.,* 2003). Furthermore, *in vivo* differentiation of bone marrow haematopoietic stem cells into *de novo* myocardium has been the most powerful evidence suggesting regenerative benefit of adult stem cells in AMI (Orlic *et al.,* 2001). This evidence for regenerative benefit was, however, challenged by *in vivo* data that suggested that bone marrow derived haematopoietic stem cells could not transdifferentiate into the component cells necessary to regenerate myocardium (Balsam *et al.,* 2004; Murry *et al.,* 2004). Interestingly, other data suggest that engrafted bone marrow derived stem cells do not transdifferentiate but acquire a cardiomyocyte phenotype by fusing with the native cardiomyocytes both *in vitro* and *in vivo* (Garbade *et al.,* 2005; Ishikawa *et al.,* 2006; Nygren *et al.,* 2004).

Non-regenerative benefits following stem cell engraftment into the native myocardium are important for the functional improvement associated with adult stem cell therapy. It is not entirely clear whether these non-

regenerative benefits exclude or enhance the regenerative benefits. Adult bone marrow derived stem cells secrete a number of anti-apoptotic factors, angiogenic factors, growth factors, and cytokines that contribute to improvements in cardiac function (Gnecchi *et al.,* 2006; Kamihata *et al.,* 2001; Kinnaird *et al.,* 2004; Uemura *et al.,* 2006). Experimental studies have shown increased myocardial blood flow and capillary density, independent of newly formed vessels after injection of bone marrow derived stem cells following AMI (Iwasaki *et al.,* 2006). These non-regenerative benefits of adult stem cell therapy might be due to up regulated angiogenic factors as secreted by the engrafted cells (Kamihata *et al.,* 2001; Kobayashi *et al.,* 2000). The secreted angiogenic factors from the engrafted bone marrow derived stem cells include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), hypoxia inducible factor-alpha (HIF- α), and angiopoietins (Takahashi *et al.,* 2006) and these could enhance neovascularization in the native myocardium (Matsunaga *et al.,* 2000; Tomanek *et al.,* 2004). Bonaros *et al.,* also report that cell therapy attenuates apoptosis of the native cardiomyocytes by enhancing neoangiogenesis and increasing blood supply to the native myocardium in a rat experimental model (Bonaros *et al.,* 2006). In addition, Gnecchi *et al.,* and Uemura *et al.,* suggested that growth factors released from the donor cells, such as VEFG, FGF, HGF or insulin-

like growth factor (IGF)-1, have an anti-apoptotic effect on the native cardiomyocyte following AMI (Gnecchi *et al.,* 2006; Uemura *et al.,* 2006).

Although it used to be considered that adult cardiomyocytes are terminally differentiated cells and have no resource for regeneration, Quaini *et al.,* identified new mature cardiomyocytes of recipient origin in the gender-mismatched transplanted human myocardium (Quaini *et al.,* 2002) suggesting that endogenous stem cells might be able to be recruited to the donor heart and differentiate into mature cardiomyocytes. This evidence encouraged experimental and clinical studies for cytokine administration, such as granulocyte-colony stimulating factor (G-CSF) or stem cell factor (SCF), to increase BM-derived circulating stem cells and consequently enhance recruitment of the stem cells into the heart to regenerate the damaged myocardium. However, data from the experimental studies are inconsistent (Dawn *et al.,* 2006; Fukuhara *et al.,* 2004) and clinical studies showed no effects of G-CSF/SCF therapy on global cardiac function following acute MI (Engelmann *et al.,* 2006; Zohlnhofer *et al.,* 2006).

Hou *et al.,* suggested that cell injection into the rat heart up regulated stromal cell derived-1 factor (SDF-1) in the myocardium (Hou *et al.,* 2005), which was reported to enhance homing of circulating stem cells into the tissue (Abbott *et al.,* 2004; Askari *et al.,* 2003) and, consequently, regenerate the damaged myocardium. Moreover, Beltrami *et al.,* and Oh *et al.,* reported the presence of residential stem cells in the adult myocardium,

which are capable of differentiating into mature cardiomyocytes both *in vitro* and *in vivo* (Beltrami *et al.,* 2003; Oh *et al.,* 2003) However, little is known about effects of cell therapy on recruitment of these endogenous stem cells into the heart.

Impairment of diastolic function is the earliest consequence of AMI, and the reduction in LV diastolic compliance is due to extracellular oedema, cellular infiltration and finally fibrosis. Fibrosis or collagen deposition within the extracellular space and hypertrophy of the non-infarcted myocardium is necessary for post AMI ventricular remodeling (Pfeffer and Braunwald, 1990). Post AMI collagen accumulation in the heart is known to be induced by an imbalance between synthesis and degradation of ECM, which is regulated by several pro-inflammatory cytokines, metalloproteinases (MMP), tissue inhibitor of metalloproteinase (TIMP) and growth factors (Spinale 2002). Ventricular remodeling improves LV function and prevents paradoxical systolic wall motion of the infarct zone. However, adverse ventricular remodeling leads to ventricular dilatation and heart failure. Experimental studies have shown that cell therapy decrease ECM collagen deposition and improve diastolic LV function of the heart post AMI (Jain *et al.,* 2001).

1.3 Aims of Thesis

The debate as to whether adult bone marrow stem cells can regenerate the heart following AMI continues. The low engraftment numbers of the exogenous adult stem cells, irrespective of the stem cell type or the modality of administration, does not favour regeneration of the ischaemia-injured myocardium. The homing mechanism of either exogenous or endogenous stem cells to the ischaemic myocardium relies upon the expression of SDF-1, which is the most powerful stem cell chemokine known to date. It therefore follows that the stem cell myocardial homing might be optimal when injecting exogenous stem cells at the peak of SDF-1 expression, i.e. at the end of ischaemia or at the beginning of reperfusion. Although this timing strategy may be optimal for stem cell homing to the myocardium, their subsequent survival may be suboptimal by exposure to the high levels of inflammatory mediators and oxidative stress typically associated with the I/R injury. The survival of the adult stem cell post myocardial homing might be relevant to their subsequent differentiation for regenerative benefits, but it is not imperative for non-generative benefits. In other words, exogenous stem cell therapy might be primarily for delivering pro-survival factors and if this significantly reduced I/R injury, then the regenerative benefit may not be necessary. The role of the vast endogenous bone marrow stem cell reservoir during AMI is yet to be been fully elucidated. There is increased mobilization of endogenous bone marrow stem cells in response to increased myocardial SDF-1

expression during myocardial ischaemia. In the absence of ischaemia, this SDF-1 cross talk with the bone marrow may not take place. Upon reperfusion, SDF-1 signaling appears to be inadequate. Nevertheless, enhanced endogenous bone marrow stem cell release co-incidental with exogenous stem cell therapy might provide additional stem cell derived benefit. The aim of this thesis is to investigate the non-regenerative benefits of adult bone marrow derived stem cells. I hypothesised that systemic IV delivery of adult bone marrow derived stem cells is cardioprotective when delivered at the onset of reperfusion. I tested this hypothesis in a Wistar male rat model of regional myocardial ischaemia and reperfusion and I investigated the following:

1. Whether IV BMMNC therapy upon reperfusion attenuates the injury caused by regional myocardial I/R (chapter 3).
2. Whether IV BMMNC therapy upon reperfusion is associated with known cardioprotective molecular mechanisms (chapter 4).
3. Whether adult bone marrow derived MSC by cell culture is cardioprotective in regional myocardial I/R (chapter 5)
4. Whether regional myocardial I/R and IPC modulate mobilisation of endogenous bone marrow mononuclear cell by SDF-1/CXCR4 signaling (chapter 6).
5. Whether combining IPC with adult bone marrow MSC therapy at the onset of reperfusion might afford better cardioprotection (chapter 7).

Chapter 2

Materials and Methods

The animal experiments described in this thesis are in adherence to (i) National Institute of Health guidelines on the use of experimental animals (ii) Home Office *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London. Male Wistar rats weighing 250-350g were maintained in a Home Office approved facility, receiving standard chow and water *ad libitum*. Test compounds are from Sigma-Aldrich (UK), unless otherwise stated.

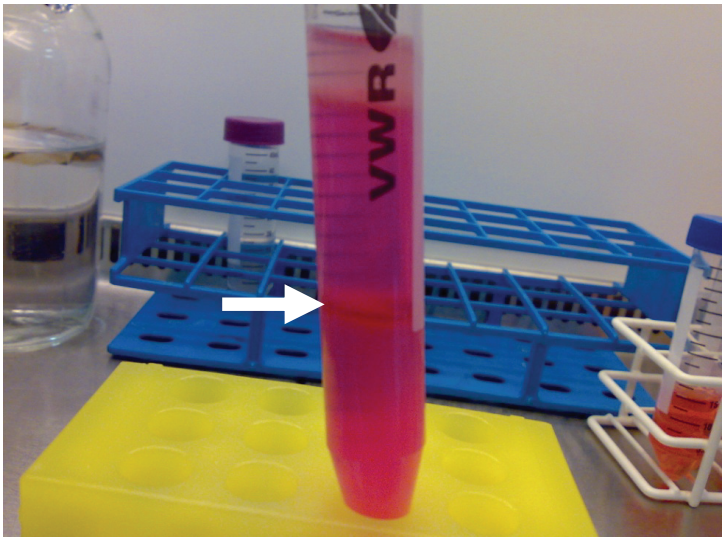
2.1 Isolation of Bone Marrow Mononuclear Cells

Adult donor bone marrow mononuclear cells (BMMNC) were isolated from whole bone marrow (BM) removed from the long bones of male Wistar rats. General anaesthesia (5% isoflurane) was prior to cervical dislocation and BM was collected from both the femurs and tibiae. The BM was collected in ice cold Hank's balanced salt solution (HBSS). Whole marrow suspension was centrifuged at 1,600 *g* for 5 min. The resulting pellet was resuspended in 1ml HBSS containing 10% v/v foetal bovine serum (FBS), penicillin 100 IU/ml, and streptomycin 100 mg/ml. This BM cell suspension were loaded on discontinuous Percoll^{TM} gradient (55% and 70%, GE Healthcare) and centrifuged at 1,600 x *g* for 30 min (Kamihata *et al.,* 2001). The interlayer containing BMMNC was collected (Figure

2.1.1A). BMMNC were washed twice with diluted 10 × volume of HBSS with 10% FBS and centrifuged at 300 x *g* for 5 min. BMMNC were then resuspended in HBSS containing 10% v/v FBS, penicillin 100 IU/ml, and streptomycin 100 mg/ml on ice until required. BMMNC viability by trypan blue exclusion test, using an equal volume of trypan blue and cell suspension incubated for 5 min at 37˚C. Then 10 µl of this mixture placed in a haemocytometer (Neubauer, VWR) to count the number of unstained (viable) and stained (non-viable) BMMNC. BMMNC viability by trypan blue exclusion was 97.05 ± 0.3%. Phenotyping of isolated BMMNC was by fluorescence activated flow cytometry (FACS) using a CyAn^{TM} ADP High-Performance Flowcytometer (DakoCytomation) equipped with Summit^{TM} software (DakoCytomation). Appropriately, 10,000-gated events were collected for univariate (log scale) histogram plots. Phenotype characterisation of BMMNC by FACS flow cytometry for the haematopoietic stem cell markers c-Kit^{+}, CD 34, CD 45, and CD 133 is shown in Figure 2.1.2.

BMMNC were washed twice with PBS to remove residual Percoll prior to re-suspension of 10 million cells in 0.5ml PBS. BMMNC administration was by a single IV bolus via a polypropylene catheter sited in the jugular vein of the animal.

A



B

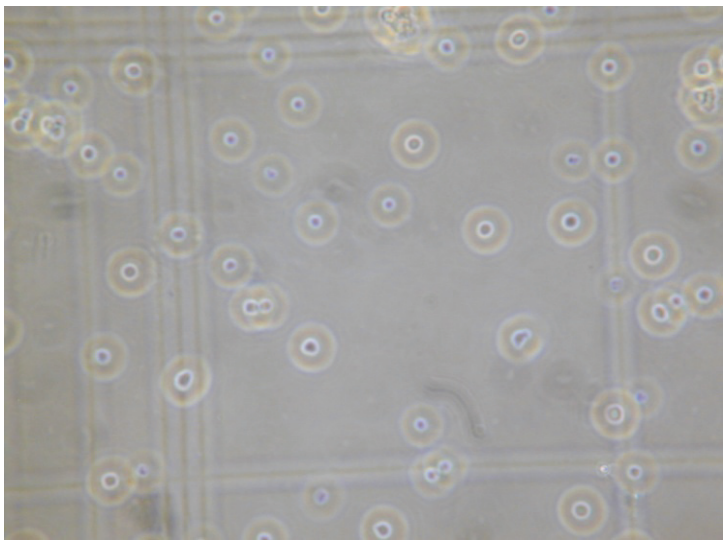


Figure 2.1.1 Isolation of adult bone marrow mononuclear cells (BMMNC) by Percoll density gradient centrifugation of *ex vivo* Wistar male rat whole bone marrow.
(**A**) BMMNC separated from other cells into the cell layer indicated by the white arrow. (**B**) BMMNC imaged by light microscopy in a haemocytometer for cell counting (mag. x 200).

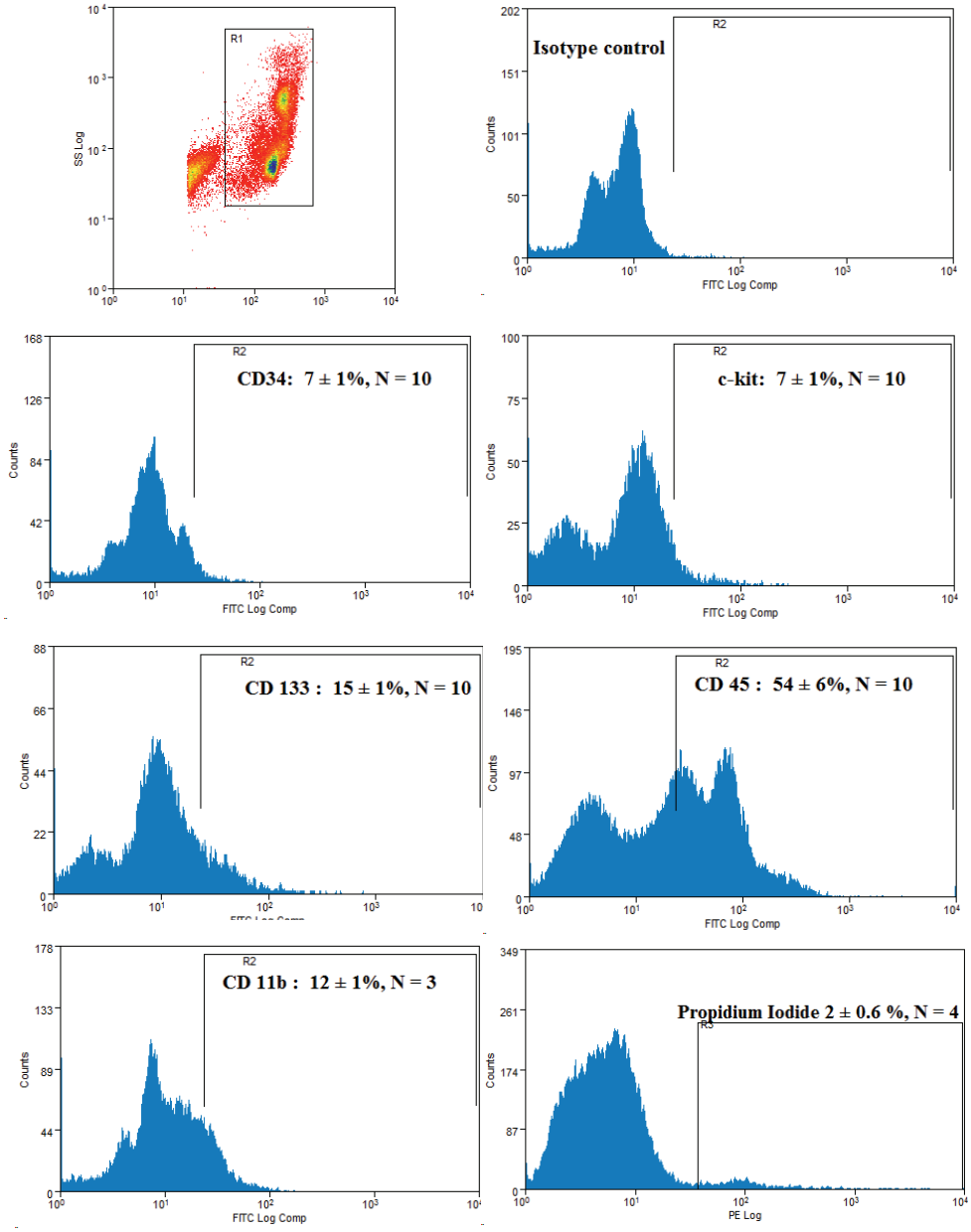


Figure 2.1.2 Bone marrow mononuclear cell (BMMNC) phenotyping by FACS flow cytometry. When compared to the isotype control antibody, BMMNC were positive for the haematopoietic stem cell markers c-Kit (7 ± 1%, n = 10), CD 34 (7 ± 1%, n = 10), CD 45 (54 ± 6 %, n =10), CD 133 (15 ± 1%, n = 10), and CD 11b (12 ± 1%, n = 3). BMMNC viability by exclusion of propidium iodide was high at 98 ± 0.6 %.

2.2 Generation of Bone Marrow Mononuclear Cell Supernatant

BMMNC supernatant (BMS) containing potential paracrine cytoprotective factors was generated by incubating a defined number of freshly isolated unfractionated BMMNC in 0.5 ml PBS. The concentrations of the BMMNC used to generate the BMS were 10 million, 20 million, 50 million, and 100 million BMMNC. The period of BMMNC incubation in PBS was for a period of either 2 h or 24 h, at 37°C in a cell culture incubator with humidified air containing 5% CO_2. Following this period of incubation, BMMNC removal was by centrifugation at 1600 x *g* and filtration through a 20 μm filter. The cell free donor BMS injection was into an IV catheter sited in the jugular vein of the experimental animal.

2.3 Isolation of Bone Marrow Mesenchymal Stem Cells

Wistar male rats euthanized by inhalation of 5% isoflurane and cervical dislocation were used to remove BM from femurs and tibiae. Following animal sacrifice, BM was collected in complete culture media: 10ml alpha-Modified Eagles Medium (α-MEM) + 1% glutamine + penicillin 100U/ml + streptomycin 100mg/ml, and 5% foetal bovine serum. The mesenchymal stem cell (MSC) isolation from the other bone marrow cells was by virtue of their adherence to a plastic surface. The BM obtained from femurs and tibae was centrifuged (1500 rpm for 5 min) and resuspended in complete culture medium for subsequent culture in T75 cell culture flasks. Non-adherent cell

removal was by repeat washes with complete culture media at the following times: 1, 2, 6, 12, 24, 48 and 72 h. The plastic adherent cell had media changes every 48 h and passage at 70% confluence by 0.25% trypsin-EDTA for 5 min in a 37^{o}C incubator with 5% CO_2. At each passage, cells' reseeding density was 1.5 x 10^5/cm^2. Culture expansion of plastic adherent cells was demonstrable beyond passage number 25, however; only cells obtained from cultures less than passage number 13 were used *in vivo* experimentation.

The mobilisation of MSC was by removal of the culture medium and incubation with 5ml 0.25% trypsin-EDTA for 5 min in a 37^{o}C incubator with 5% CO_2. The disassociated cells were collected in 1ml Hank's Buffered Salt Solution (HBSS), centrifuged 1500 rpm for 5 min, and resuspended in 100 µl HBSS.

Culture expanded plastic adherent cells were phenotyped for expression of haematopoietic lineage markers (CD 34, c-Kit, CD 45, CD 133); matrix receptors (CD 44); integrins (CD 29, CD 90). Cell viability was by exclusion of propidium iodide. Primary antibody for each cell surface marker was by incubation of one million cells with monoclonal antibody for 30 min on ice. Washing the cells with 1 ml HBSS, centrifugation (1500 rpm for 5 min), resuspension in 100 µl HBSS, and repeat removed unbound antibody. Secondary antibody binding to primary antibody was by incubation for 30 min on ice and washing twice with HBSS (1ml). Cells are

resuspended in 500µl 2% paraformaldehyde (PFA) in PBS and analyzed by Fluorescence activated cell sorting (FACS) flow cytometry using a DakoCyan flowcytometer equipped with Summit v4.3 software (Dako, UK).FACS data collected 10,000 cellular events using 3-decade logarithmic amplification. FACS phenotyping of MSC is presented in Figures 2.3.2 & 2.3.3.

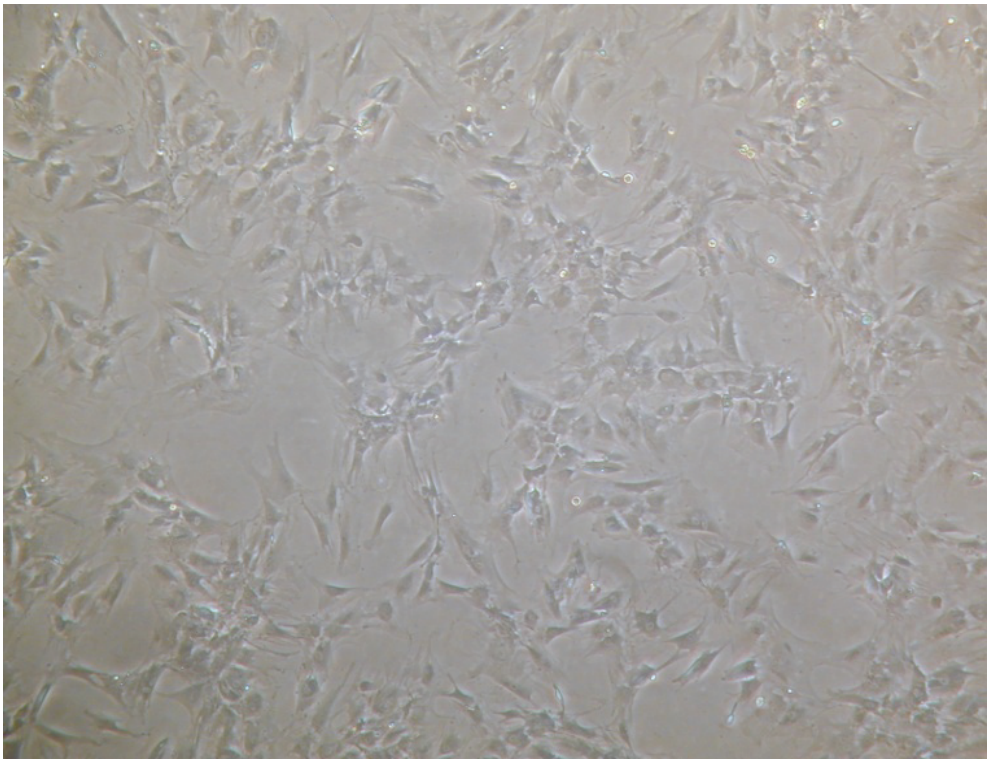


Figure 2.3.1. Adult mesenchymal stem cells (MSC) isolated from *ex vivo* Wistar male rat whole bone marrow and expanded in culture. This light microscope image demonstrates plastic adherent MSC with a typical fibroblast appearance and confluent growth in a T75 cell culture flask (mag x100).

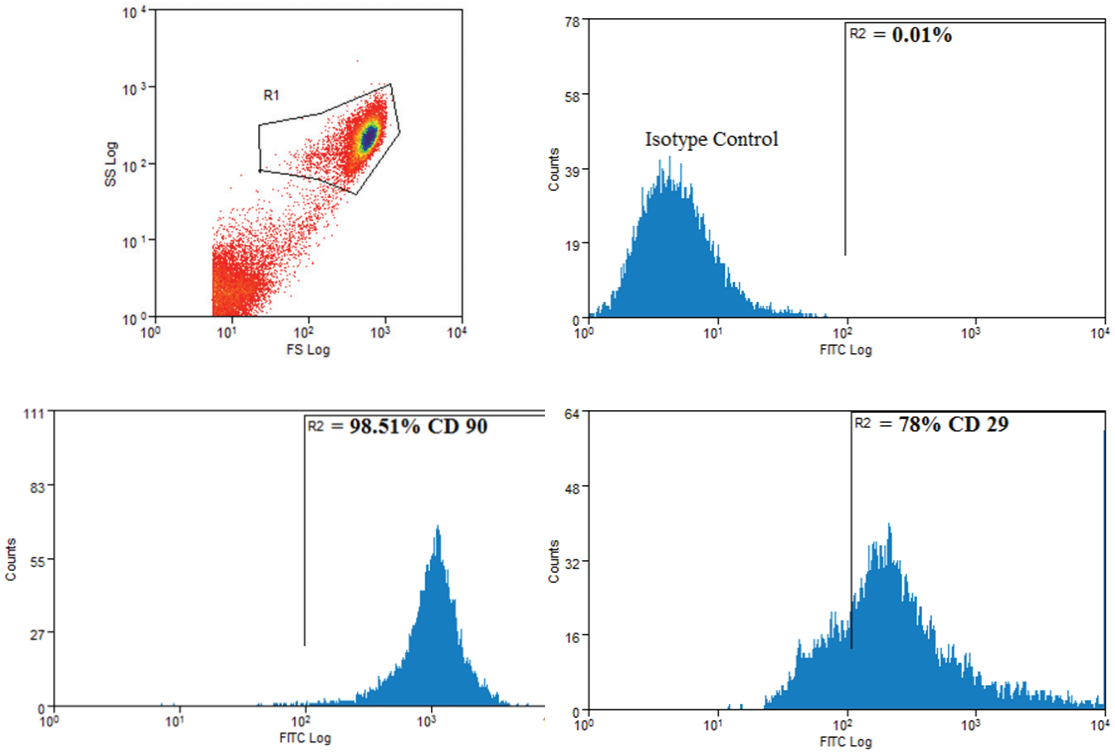


Figure 2.3.2 Culture expanded adult bone marrow derived mesenchymal stem cell (MSC) phenotyping by FACS flow cytometry for the R1 gated cell population as shown in the scatter plot. MSC isolated from BM and following culture expansion were phenotyped at passage 3. When compared with the isotype control antibody, MSC were positive for CD 90 and CD 29.

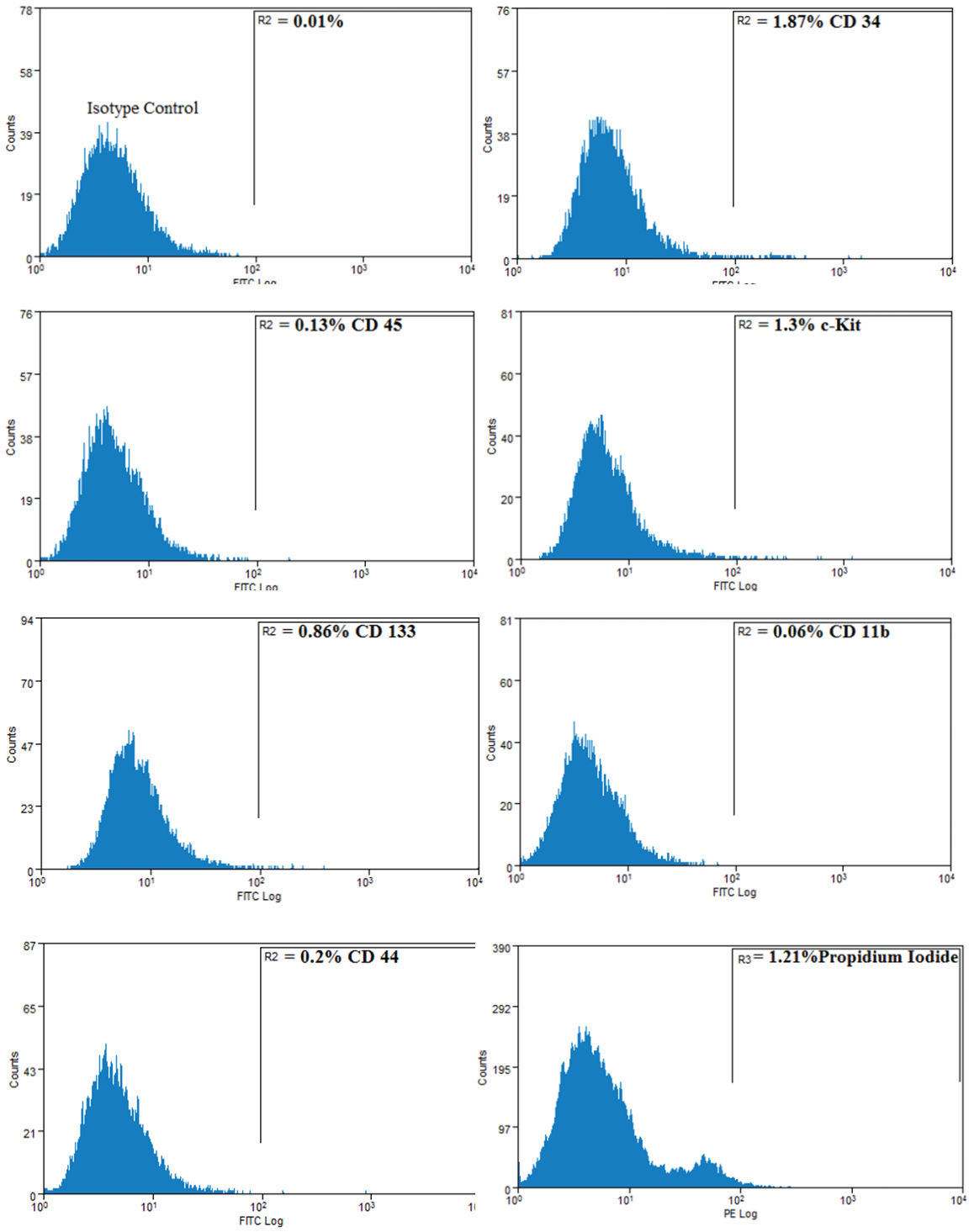


Figure 2.3.3 Culture expanded adult bone marrow derived mesenchymal stem cell (MSC) phenotyping by FACS flow cytometry following expansion to passage 3. When compared with the isotype control antibody, MSC were negative for the haematopoietic stem cell markers CD34, c-Kit, CD133, CD45, CD44, and CD11b. MSC viability by exclusion of propidium iodide was high at 98.8%.

2.4 Regional Myocardial I/R

Anaesthetised male Wistar rats were subjected to LAD ischaemia and reperfusion (I/R). General anaesthetic was with thiopentone (Intraval® 120 mg/kg i.p.). Tracheal intubation was necessary for ventilation of the animals with a small animal ventilator (Harvard Apparatus Ltd., Edenbridge, Kent. U.K.), so that thoracic entry for access to the heart was possible without loss in effective ventilation. The ventilator settings: 30% inspired oxygen, at a rate of 70 mandatory ventilations per min, and a tidal volume of 8-10 ml/kg. The body temperature monitoring was with a rectal probe thermometer and maintenance at 37±1^{o}C was with a homoeothermic blanket unit (Harvard Apparatus Ltd., Edenbridge, Kent. U.K.). A right carotid artery cannulation was necessary for mean arterial pressure (MAP) and heart rate (HR) transducer monitoring (Senso-Nor 840, Senso-Nor, Horten, Norway) with a data acquisition system (Mac Lab 8e, ADI Instruments, Hastings, UK) in line with a Dell Dimension 4100 desktop computer. Right jugular vein cannulation was necessary for IV administration of fluids. The heart exposure was via a left thoracotomy incision. The pericardium dissected from the heart to expose the left anterior descending (LAD) coronary artery and snared proximally with a 7-0 mersilk suture (Ethicon, UK). The LAD occlusion begins, after having achieved haemodynamic stabilization and acclimatization over a 30 min period, at time 0 with a snare occlusion device (Figure 2.4.1). The LAD territory ischaemia confirmation was by anterior LV visual colour changes and ST segment elevation of the

electrocardiogram (Figure 2.4.1B). Following the 25 min period of LAD occlusion, LAD ligature release allows reperfusion to commence and continue for a defined period. The MAP and HR was noted from baseline (prior to onset of myocardial ischaemia) and throughout regional myocardial I/R. The pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (Baller *et al.,* 1980), is a product of MAP and HR and expressed in mmHg $min^{-1}10^{-5}$. Boluses of IV saline (1 ml/kg/h) via the central venous cannulation were at every 30 min intervals throughout reperfusion.

The generic regional I/R protocol and BM derived stem cell therapy by IV injection upon reperfusion is shown in Figure 2.4.2.

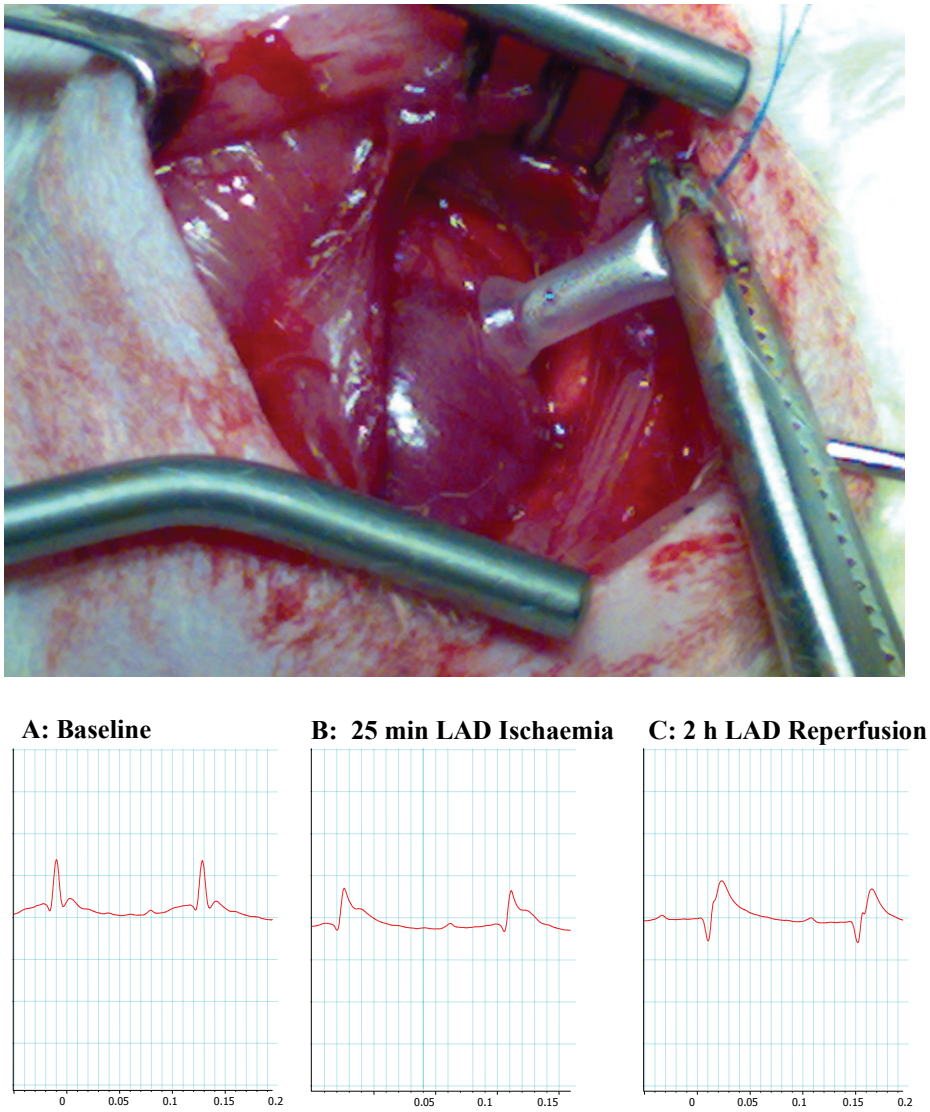


Figure 2.4.1 An anaesthetized male Wistar male rat with the heart exposed and subjected to LAD occlusion. The LAD territory myocardial ischaemia confirmation was by anterior LV immediate cyanotic colour change and the ST segment elevation, which is notable in the representative electrocardiograms from **A** to **B**. At end of 2 h reperfusion, transmural infarction was notable by Q-wave development in the electrocardiogram shown in **C**.

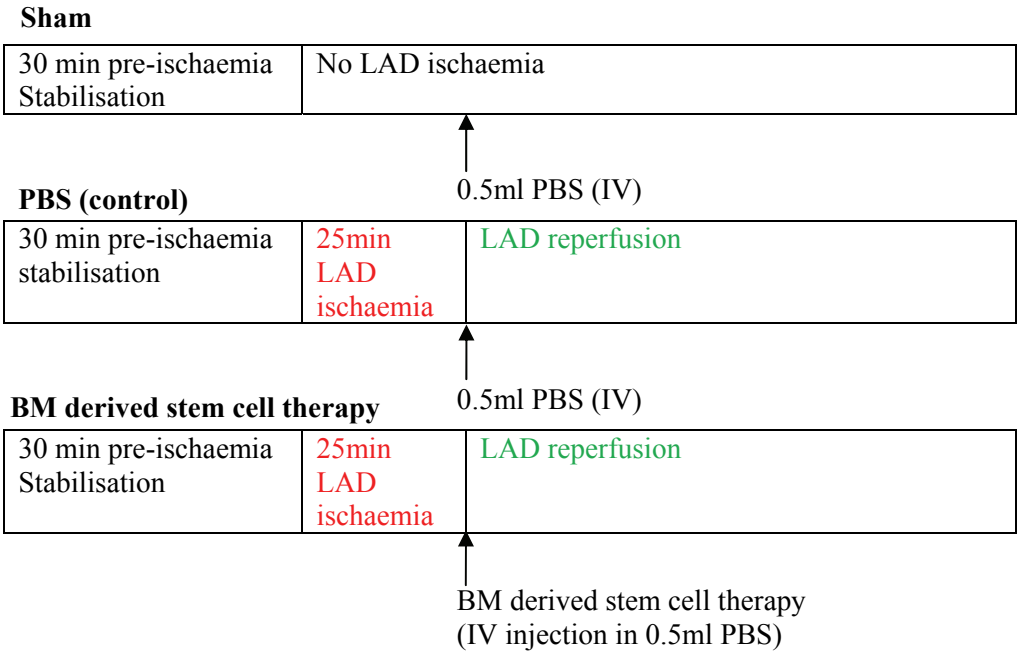


Figure 2.4.2 Generic regional I/R protocol and BM derived stem cell therapy by IV injection upon reperfusion. The generic Wistar male rats myocardial regional I/R protocol. Following general anaesthesia and surgical manipulation a 30 min of pre-ischaemia stabilisation was allowed, which was proceeded by 25min LAD occlusion and a defined period LAD reperfusion. For the specific period period of reperfusion see methods section the relevant chapter. Sham animals underwent the same anaesthesia and surgery but no LAD occlusion (I/R).

2.5 Myocardial Infarct Size post Regional I/R

Following regional I/R, the identification of the LAD territory subjected to I/R was by LAD re-occlusion and injection of Evans Blue dye (1 ml of 2% w/v) via the right internal jugular vein cannulation. The Evans Blue solution stains the perfused myocardium, while area of the LV subjected to I/R (area at risk) remains unstained. Immediately, after identification of the area at risk (AAR) the animal was euthanized with an overdose of anaesthetic to excise the heart. The explanted heart was then immediately subjected to transverse or short axis 3–4 mm slices, to excise the unstained AAR from the blue stained non-ischaemic myocardium, which included the right ventricle and part of the left ventricle. The AAR was further cut into small pieces for identification of viable from non-viable myocardium by incubation of the AAR with 0.5mg/ml *p*-nitro blue tetrazolium (NBT) for 20 min at 37°C. Viable myocardium with intact dehydrogenase enzyme systems converts the NBT into a dark blue formazan, whilst the infarcted AAR lack dehydrogenase activity and therefore fails to stain (Nachlas *et al.,* 1963). The weight of unstained AAR was expressed as a percentage of the total weight of the AAR, which is the myocardial infarct size or the primary end-point.

2.6 Isolation of Cardiomyocytes post Regional I/R

Cardiomyocyte isolation from the LV region post I/R was as previously described (Mitra *et al.,* 1985), with some modifications. Following 25 min LAD occlusion and 2 h reperfusion, the whole heart was explanted and perfused with 20 ml cold (0-4˚C)antegrade coronary cardioplegia solution at a rate of 2 ml/min via a cannula placed in the ascending aorta. Immediate cardiac arrest was achieved using ice-cold cardioplegia bicarbonate buffer (CBB) solution (AQIX® RS-I). Next perfusate was warm 20 ml (37˚C) CBB containing collagenase (Gibco) and protease, via the aortic cannula. Final perfusate was 20 ml warm (37˚C) CBB containing protease inhibitor was via the aortic cannula. The area of the left ventricle at risk from I/R injury (AAR) was demarcated by re-occluding the LAD and injecting Evans™ blue antegrade via the aortic cannula. The unstained AAR was excised, finely diced and gently pressed through a 300 μm steel sieve to release the cells. The AAR cell suspension was centrifuged and washed twice with cold CBB solution (AQIX® RS-I). The isolated AAR cells were suspended in cold buffer solution (AQIX® RS-I) and stored on ice, prior to staining for subsequent fluorescent-activated cell sorting (FACS) flow cytometric analysis. Figure 2.6.1 shows isolated cardiomyocytes from the AAR as seen under light microscopy.

Isolated AAR cell binding to a Troponin-T monoclonal antibody was also checked to ensure that the isolated cells were indeed cardiomyocytes.

Incubation of the putative cardiomyocytes with a primary mouse monoclonal antibody for cardiac Troponin-T (Santa Cruz, USA), was for 30 min on ice. Unbound antibody removal was by washing the cells twice and then incubation with a goat anti-mouse antibody conjugated with fluorescein isothiocyanate (Santa Cruz, USA). Figure 2.6.2 shows the FACS flow cytometry analysis of isolated cardiomyocytes for the Troponin T binding.

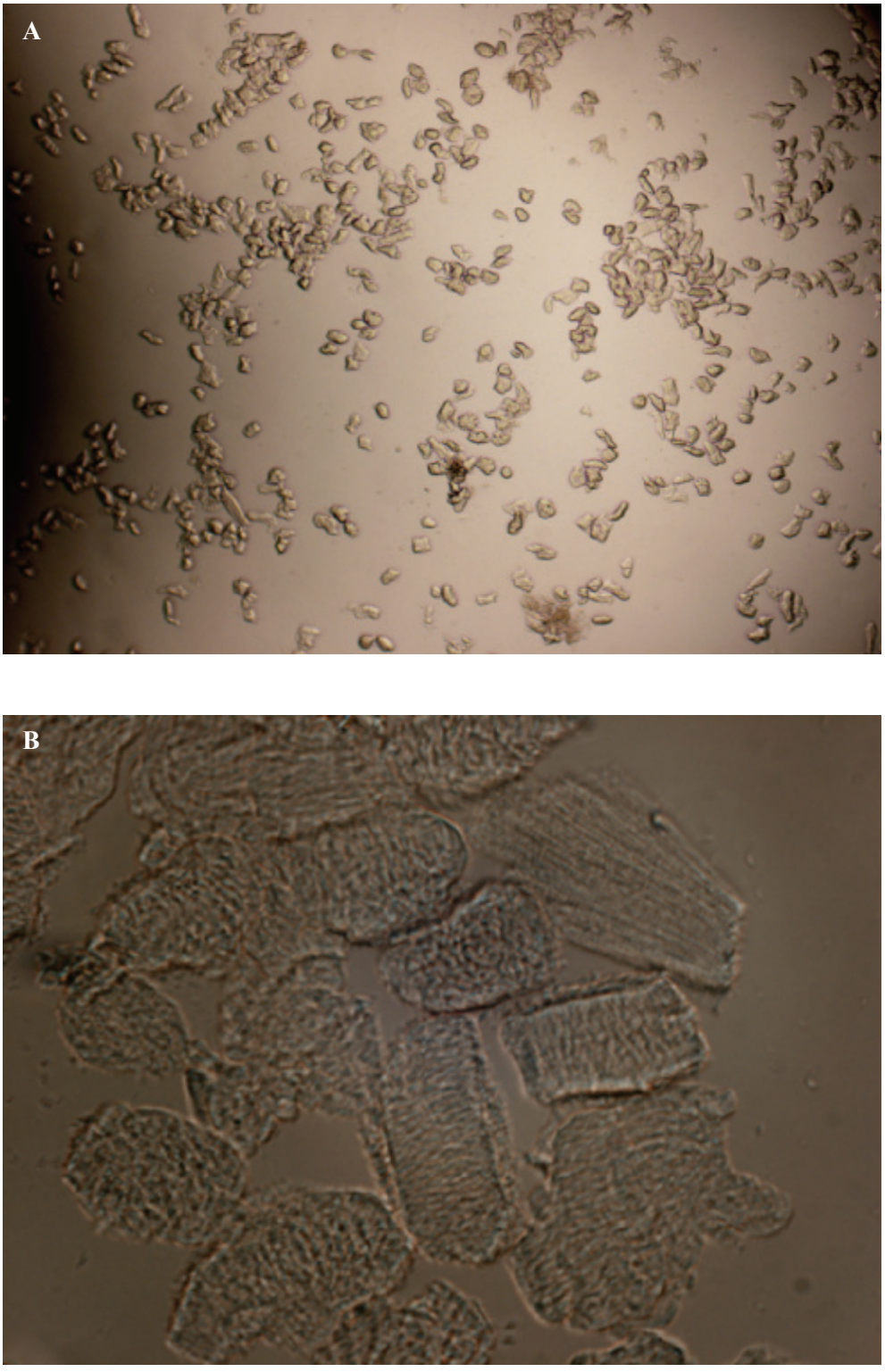


Figure 2.6.1 Isolated cardiomyocytes (A) lower power (mag x 200) and (B) high power microscopy (mag x400)

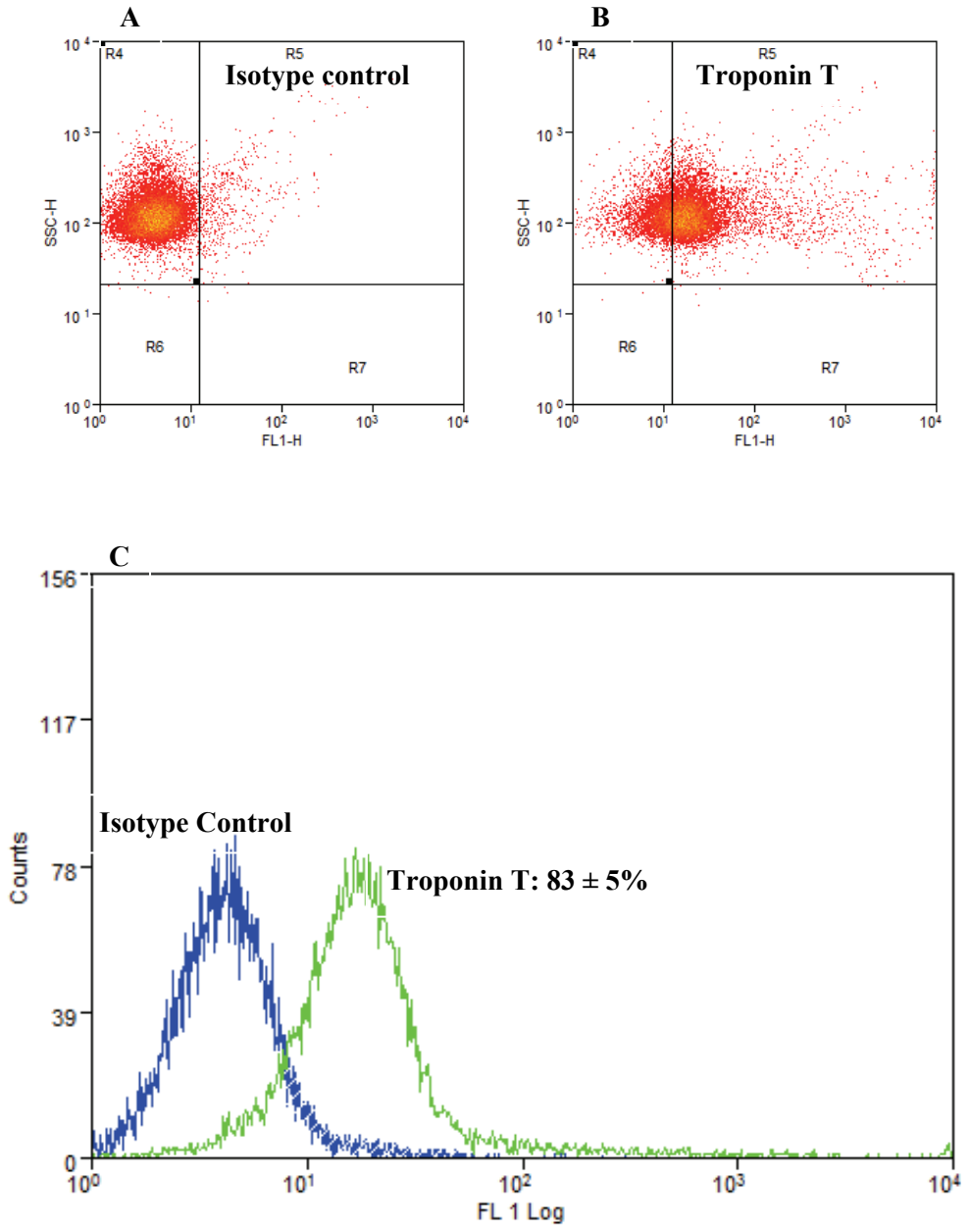


Figure 2.6.2 FACS flowcytometry of isolated cardiomyocytes. (A&B) Respective FACS flow cytometry scatter plots of side scatter versus FL1 for cardiomyocytes stained with the isotype control and Troponin T antibodies both conjugated with FITC. (**C**) Overlay histogram plot for cardiomyocytes stained with isotype control and Troponin T antibodies conjugated with FITC.

2.7 Cardiomyocyte Apoptosis and Necrosis post Regional I/R

An apoptosis detection kit was used to dual stain cardiomyocytes isolated from the AAR with Annexin-5-FITC and propidium iodide, as per kit instructions (BD Biosciences Pharmingen). Unstained cells were negative controls. The AAR cardiomyocyte staining and analysis was within 1 h of cell isolation. Dual stained cell analysis was by a Becton Dickinson FACScan flow cytometer (BD FACScan, UK) and equipped with *Cell-Quest* software (BD). The flowcytometer gating was set to collect 10,000 cells from each sample with logarithmic gain settings. Annexin-5 and propidium iodide cannot permeate an intact plasma membrane and so will not stain viable cells. Annexin-5 will avidly bind to the inner membrane phospholipid phosphatidylserine, which becomes externalised by cells undergoing apoptosis. Cells that have lost membrane integrity will not only stain positive for Annexin-5 but they will concomitantly stain with propidium iodide, which binds to nuclear deoxyribonucleic acid (DNA). Dual staining of AAR cardiomyocytes with annexin-5-FITC and propidium iodide was analyzed by FACS flow cytometry to identify cells that were either apoptotic (annexin-5^{+}/PI^{-}) or necrotic (annexin-V^{+}/PI^{+}). Quadrant percentiles for apoptosis and necrosis.

Detection of activated caspase 9 activation in AAR cardiomyocytes was to distinguish between apoptosis by intrinsic or extrinsic mechanisms. AAR cardiomyocytes stained with caspase 9 assay kit based upon

Fluorescent Labeled Inhibitor of Caspases (FLICA) reagent FAM-LEHD-FMK, which is a non-toxic membrane permeable fluorescent probe. The LEHD peptide sequence within the FLICA binds avidly to and inhibits activated caspase 9. Bound FLICA will remain inside the cell to give a direct measurement of the number of active Caspase 9 molecules that were present at the time of addition of FLICA reagent. Unbound FLICA is easily washed from either necrotic or viable cells. FACS flow cytometry detects the fluorescent FAM moiety bound to FLICA and so quantifies activated Caspase 9.

2.8 Cardiac Function post Regional I/R

The cardiac function post regional I/R was assessed at the end of 7 days of reperfusion (I/R-7D). The protocol for regional I/R was altered to enable recovery of the animals and this is briefly described. Animals were anaesthetised by inhalation of 1.5% isoflurane and mechanical ventilation was commenced following endotracheal intubation at a tidal volume of 200 ml/min using a Harvard small animal ventilator. The heart exposure was via a left thoracotomy and the LAD was isolated with a 6-0 polypropylene suture (Bear Medic). The LAD occlusion was with a reversible occluder for 25 min, and anterior regional ischemia confirmed by an ischaemic colour change and S-T segment elevation on the electrocardiograph. Following 25 min LAD occlusion, the occluder release allows reperfusion, which continues for 7 days. The thoracotomy closure was with a 4-0

polypropylene suture (Bear Medic), and then the termination of the anaesthetic allowed spontaneous post procedural recovery. Post recovery animals received prophylactic antibiotics and buprenorphine analgesia in their water. A standard diet was resumed upon return to the animal facility.

Transthoracic 2-dimensional (2-D) echocardiography was used during general anesthesia using a Vevo-770 imaging system with a 17.5MHz small animal transducer probe (Vevo, Visual Sonics, USA). The anaesthetic (1.5% isoflurane) delivery was via a nose cone. Echo images acquisition was on the day before I/R and 7 days post I/R. Short axis views at the mid-papillary muscle level were used for assessment of the fractional area of LV contraction (% FAC). The LV ejection fraction (LVEF) assessment was by motion mode (M-mode).

Haemodynamic LV catheter analysis was performed under anesthetic (1.5% isoflurane) delivered via a nose cone, on the day before and 7 days post I/R. A 2-Fr micro tipped pressure transducer (Millar Instruments; SPR-320) was inserted into the LV cavity, via the right carotid artery, for measurement of LV pressures during the cardiac cycle. The following LV pressure were noted: peak systolic pressure (LVPSP), end diastolic pressure (LVEDP), maximal slope of systolic pressure increment (+dP/dt), diastolic pressure decrement (−dP/dt), and the relaxation time constant (tau). The LV pressures and the heart rate were analyzed using LabChart Pro software (AD Instruments, UK).

2.9 Myocardial Fibrosis post Regional I/R

Explanted hearts subjected to regional ischaemia and 21 days reperfusion were perfusion fixed with 1 ml ice-cold 4% paraformaldehyde via the aorta and then immersed in ice-cold 4 % paraformaldehyde for 30 min. Hearts were then washed with PBS and incubated in PBS containing 30% sucrose (w/v) at 4°C overnight. Fixed hearts were then sliced transversely into three pieces, embedded in OCT compound (BDH), and then frozen in liquid nitrogen-cooled isopentane for storage at -80°C. Subsequently cryosections (15-25 μm) from each segment were made using a rotary cryotome (Leica). Cryosections were placed on polysine-coated glass slides (VWR) and stained with 0.1% picrosirius red F3B (0.1 g Sirus red in 100 ml saturated aqueous picric acid, BDH) for 10 min at room temperature. The cryosections were rinsed 5 times in deionised water and then rinsed for 1 min in picric alcohol (20 ml absolute alcohol; 70 ml deionised water; 10 ml saturated aqueous picric acid). The sections were then dehydrated through a methanol series and mounted in DPX (VWR). The degree of picrosirius red staining was visualised by a light microscope (Keyence, BZ8000).

2.10 Cell Signaling

The AAR from 25 min LAD ischaemia followed by only 30 min reperfusion underwent analysis for cell signaling. AAR from the following animal groups were analysed by western blotting: (1) pre-treatment with LY294002 (0.3 mg/kg IV) followed by sham surgery. (2) Pre-treatment with PBS followed by 25 min LAD ischaemia and 30 min reperfusion with treatment upon reperfusion with either PBS or BMMNC. (3) Pre-treatment with the PI3K inhibitor LY294002 (0.3 mg/kg IV) followed by 25 min LAD ischaemia and 30 min reperfusion with treatment upon reperfusion with either PBS or BMMNC. The *ex vivo* AAR samples are snap frozen in liquid nitrogen and stored at -80 °C, for subsequent protein analysis by western blots.

The protein extracts from AAR investigated for cell signaling extracted were from cytosolic and nuclear fractions, as described (Meldrum *et al.,* 1997). Briefly, AAR was homogenized (Wheaton, Millville, NJ, USA) 10% w/v in homogenization buffer containing 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM EDTA, 1% NP–40, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), 5 µg/ml aprotinin, 2.5 µg/ml leupeptin. Homogenates centrifuged at 1000 × *g* for 5 min at 4 °C. Supernatants were removed and centrifuged at 105,000 × *g* at 4°C for 40 min to obtain cytosolic protein fractions. Pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 300 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM

EGTA, 1 mM DTT, 0.5 mM PMSF, 5 μg/ml aprotinin, 2.5 μg/ml leupeptin. The suspensions were incubated on ice for 30 min for high-salt extraction followed by centrifugation at 15,000 × *g* for 20 min at 4°C. The resulting supernatants containing DNA-binding proteins were carefully removed, protein content was determined using the Bradford assay (Bradford 1976) and samples were stored at − 80 °C. Western blots of the cytosolic protein extracts for phosphorylation of Akt, GSK-3β, and p38 MAPK were as previously described (Collino *et al.,* 2006). Western blots for the nuclear translocation of p65 NF-κB was for protein extracts from both the cytosolic and nucleus fractions. Briefly, electrophoresis of 15μg of total protein extract was on 8% sodium dodecyl sulphate-polyacrylamide gel (SDSPAGE) followed by transfer to a polyvinyldenediflouride (PVDF) membrane (Millipore, USA) for subsequent immersion in blocking buffer (Super Block, Pierce Biotechnology, USA). PVDF membrane incubation are then with the following a primary antibody. The primary antibodies were: mouse anti-rat phosphorylated $Akt^{serine\text{-}473}$ (Cell Signaling Biotechnology, USA); rabbit anti-rat total Akt; rabbit anti-rat total GSK-3β (Santa Cruz, California, USA); goat anti-rat phosphorylated $GSK\text{-}3\beta^{Serine\text{-}9}$ (Santa Cruz,USA); goat anti-rat phosphorylated $GSK\text{-}3\beta^{Tyrosine\text{-}216}$ (Santa Cruz); mouse anti-rat phosphorylated p38 MAPK (Cell Signaling Biotechnology, USA); and mouse anti-rat NF-κB p65 (Santa Cruz). Primary antibody binding detection was by incubation with appropriate secondary antibodies conjugated with horseradish peroxidase and development with an

enhanced chemiluminescence detection system (Amersham, UK). Assessment for loading homogeneity was by incubation of the stripped membrane in β-actin monoclonal antibody and subsequently with anti-mouse antibody. Membranes we evaluated for reactive immune bands by and quantification of band optical density was by Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, USA).

2.11 Proteomics post Regional Myocardial I/R

Proteomics gives a quantitative analysis of all the proteins, including their post-translational modifications, expressed by the genome at any given point in time (Wilkins *et al.,* 1996). Proteomic data could therefore provide valuable insight into altered protein expression and associated pathology (Huber *et al.,* 2003; Lopez *et al.,* 2002; Loscalzo 2003; Mcgregor *et al.,* 2003).Thus the differential expression of proteins extracted from the ischaemic and reperfusing myocardium can give insight into underlying mechanisms of injury and protection. The resolution of proteins by 2-dimensional gel electrophoresis (2-DE) remains the preferred method to date (Gorg *et al.,* 2004). 2-DE can resolve up to 5000 proteins simultaneously with detection and quantification of <1ng of protein per spot. There is no current technological match for 2-DE, which permits routine parallel expression profiling of a large complex of proteins. It delivers a map of intact proteins that reflect changes in protein expression level, isoforms, and post-translational modifications. I investigated the

effect of BMMNC and BMS on the differential expression of proteins within the area at risk (AAR) of the LV subjected to 25 min ischemia and 2 h reperfusion (I/R-2h).The AAR analyzed was from hearts treated upon reperfusion by, PBS, BMMNC, BMS or sham (no I/R-2h). At the end of I/R-2h, the excised AAR was snap frozen in liquid nitrogen and stored at -80°C, for later proteomic analysis.

Protein extraction from thawed AAR tissue was previously described (De Roos *et al.,* 2005). Briefly, proteins were extracted from 300mg of tissue and 1.2ml of extraction buffer (Ultrapure Urea 40% w/v, Thiourea 15.2% w/v, CHAPS 4% w/v, Ampholyte 5% v/v; Bio-Rad, USA). Tissue from AAR was homogenised and then sonicated for 3 x 10 seconds periods with 3 min intervals between each sonication to allow cooling. Then samples were centrifuged at 14000 rpm in Eppendorf tubes and proteins in the supernatant were then separated according to size and charge by 2-DE in SDSPAGE gels, as previously described (De Roos *et al.,* 2005). Separated proteins spots, after staining with Coomassie Brilliant Blue, were scanned (GS-800 calibrated densitometer, BioRad, USA) and analyzed for differential protein spot expression (PDQuest Advanced 8.02, Bio-Rad, USA). Then an automated robotic protein handling system, which excised selected gel spots, with optical densities that were significantly different, for trypsinisation (EXQuest Spot Cutter and MassPREP^{TM}, Bio-Rad USA). Trypsinised protein spot analysis was by liquid chromatography, electrospray ionisation, and tandem ion-trap mass spectrometry/mass

spectrometry (LC/ESI/MS/MS, Q-Trap 4000, Applied Biosystems, UK). The high performance liquid chromatography (HPLC) system was equipped with a 100-angstrom nanocolumn measuring 15 cm x 75 μm x 3 μm, (C18 Pep Map 100, LC Packings, Camberly, Surrey, UK). Sample injection into the column by a Famos auto sampler was set to an injection volume of 5 μl and the column flow rate was 0.3 μl/min. The samples were eluted with HPLC grade solvents including 2% acetonitrile and 0.1% formic acid (A) and 80% acetonitrile and 0.08% formic acid (B). The column gradient started at 5% solvent B, increasing to 50% B over 30 min followed by a ramping up to 80% B over a further 2 min and held for a further 10 min. The system was equilibrated with 95% solvent A for 9 min prior to injection of peptides samples. HPLC column eluted peptides into the Q-Trap triple quadrupole mass spectrometer (MS). The MS was fitted with a nanospray ion source and the needle voltage was set at 2450 volts, with oxygen free nitrogen as the curtain gas and the collision gas. In the survey scan mode, the mass range was set to 400-1200 (mass/charge ratio) with a scan rate of 4000 atomic mass units/second (amu/s). Criteria for selection of ions for fragmentation were set for ions of 10^5 counts per second or above. The collision energy was set to a maximum of 80 electron volts. The linear ion trap fill time was 250 milliseconds and the scan rate was 1000 amu/s. Total ion current data was submitted online to the MASCOT search engine (Matrix Science) using the MSDB database. The following search criteria were used : allowance of 0 or 1 missed cleavages; peptide mass tolerance of

± 1 Daltons (Da) ; fragment mass tolerance of ± 0.8 Da, trypsin as digestion enzyme; carabamidomethyl modification of cysteine; and methionine oxidation as partial modification.

2.12 Statistical Analysis

Prism 5 statistics package (Graphpad, USA) was used to analyse the results. All data presentation was means ± SEM, and analysed by one-way ANOVA followed by Dunnett's post test for multiple comparisons. In the comparison of just two groups, Student's unpaired t-test was used. Haemodynamic parameters analysis was by two-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. A two-tailed P value < 0.05 was considered to be statistically significant. For proteomic data, the pooled variance of the protein spots were compared by ANOVA and post-hoc unpaired t-tests.

Chapter 3

Bone Marrow Mononuclear Cell Therapy upon Reperfusion Attenuated Regional Myocardial I/R Injury

3.1 Introduction

The most effective route and optimal time point for the delivery of stem cells to an acutely ischaemic heart is still debatable. Stem cell injection to target the heart can be by (1) systemic intravenous (IV) i.e. systemic or retrograde coronary (2) intra-coronary (IC) (3) intra-myocardial (IM). Injection of cells by IM or IC routes suggests that high numbers of stem cells might directly target areas of the heart where stem cell therapy may be beneficial, however, significant risks may outweigh potential benefits. These risks include clustering or islands of stem cells within the myocardium following IM injection, which may interfere with electromechanical synchrony and trigger dangerous cardiac arrhythmias (Leobon *et al.,* 2003; Reinecke *et al.,* 2002). Despite the advantage of a high initial deposition of stem cells following IM injection, these cells are rapidly lost from an ischaemic myocardium (Muller-Ehmsen *et al.,* 2006). The antegrade IC injection also potentially delivers a high number of cells directly into the myocardial micro-circulation and relies on stem cell transmigration abilities to extravasate into the myocardium. Failure to

transmigrate, however, carries a high risk of intracoronary stem cell aggregation and distal embolization, which could compromise coronary perfusion and negate the potential benefits of cell therapy, particularly in the reperfusing heart (Suzuki *et al.,* 2000; Vulliet *et al.,* 2004). On the other hand, IV injection delivers a comparitively smaller number of donor cells directly to the heart due to the dilutional effects of the circulating volume and secondly due to extra-cardiac organ capture by the lungs, lymphatic tissue, and the bone marrow. There are, however, clinical advantages of IV delivery over that by IM and IC, which include a minimally invasive route of delivery, wide availability, comparatively low technical skill requirement for peripheral IV cannulations. Further, if the timing of stem cell delivery by IV coincides with the highest expression of SDF-1within the myocardium, i.e. at the end of ischaemia, exogenous stem cells could preferentially traffick down the SDF-1 gradient to the ischaemic myocardium. It is noteworthy, that this might not be the case if the cell delivery is pre-ischaemia, in the absence of reperfusion, or if a long period of reperfusion has elapsed. This may explain, at least in part, why the clinical trials investigating autologous bone marrow mononuclear cell (BMMNC) therapy in AMI patients report very small improvements in cardiac function. Specifically, BMMNC delivery was typically 24 h after culprit coronary artery reperfusion (Chen *et al.,* 2004; Janssens *et al.,* 2006; Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006).

BMMNC are easily harvestable and thus have been in investigated in clinical trials for treating AMI (Chen *et al.,* 2004; Janssens *et al.,* 2006; Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006). BMMNC contain primarily haematopoietic stem cells and their ability to regenerate regions of damaged myocardium, is controversial (Balsam *et al.,* 2004; Jackson *et al.,* 2001; Murry *et al.,* 2004; Orlic *et al.,* 2001). Further, BMMNC therapy in AMI might be improving cardiac function by secreting growth factors, angiogenic factors, and anti-apoptotic factors (Gnecchi *et al.,* 2006; Kamihata *et al.,* 2001; Uemura *et al.,* 2006; Kinnaird *et al.,* 2004). In this chapter, I investigated the effect of systemic IV syngeneic *ex vivo* BMMNC at the onset of reperfusion upon infarct size, cardiac function, and cardiac fibrosis in a rat model of regional myocardial I/R.

3.2 Intravenous BMMNC Therapy upon Reperfusion and Myocardial Infarct Size

3.2.1 Methods

Donor BMMNC were freshly isolated from *ex vivo* BM expressed from the femurs and tibae of Wistar male rats, not subjected to regional myocardial I/Rinjury, as described in chapter 2. BMMNC were positive for c-Kit (7 ± 1%, n = 10), CD34 (7 ± 1%, n = 10), CD45 (54 ± 6 %, n =10), and CD133 (15 ± 1%, n = 10). BMMNC cell viability was 97.1 ± 0.3% (n = 10).

Randomly selected Wistar male rats were subjected to regional myocardial ischaemia and reperfusion, by 25 min of LAD occlusion followed by 2 h of LAD reperfusion (I/R-2h), as described in chapter 2. Sham rats are subject to the same surgical procedure but not LAD occlusion and reperfusion. IPC was by two consecutive cycles of 5 min LAD ischaemia followed by 5 min LAD reperfusion, prior to the I/R-2h. Animals received either 0.5 ml PBS or 10 million BMMNC (in 0.5 ml PBS), via the internal jugular vein at the onset of reperfusion. At the end of reperfusion, infarct size measurement was by the method described in chapter 2 (Nachlas *et al.,* 1963). The experimental groups are as indicated Table 3.2.1.1

Table 3.2.1.1 Experimental Design: BMMNC therapy and infarct size following 25min LAD ischaemia and 2 h reperfusion. Wistar male rats underwent either 25 min LAD occlusion and 2 h of LAD reperfusion (I/R-2h) or no LAD occlusion i.e. sham procedure. IPC was by two consecutive cycles of 5 min LAD ischaemia followed by 5 min LAD reperfusion, prior to the I/R-2h. The n value is the number of animals per group.

Group	**N**	**Reperfusion Protocol**	**IV injection upon Reperfusion**
Sham	5	No I/R-2h	0.5 ml PBS
PBS	5	I/R-2h	0.5 ml PBS
IPC	9	IPC then I/R-2h	0.5 ml PBS
BMMNC	10	I/R-2h	10 x10^6 BMMNC (0.5 ml PBS)

3.2.2 Results

In Figure 3.2.2.1(A), left ventricular AAR from I/R-2h was similar between all the experimental groups. As shown in Figure 3.2.2.1(B), when compared with PBS (vehicle controls), IV bolus administration of 10 million BMMNC upon reperfusion significantly attenuated myocardial infarct size due to I/R-2h (BMMNC 33 ± 3 %; PBS 57 ± 2 %, 1-way ANOVA and Dunnett's post test, $P < 0.001$). Infarct size comparisons between BMMNC and IPC were, however, similar ($P>0.05$). The attenuation of infarct size by either BMMNC or IPC was independent of any effects due to variations in haemodynamic parameters between the groups as presented in Figure 3.2.2.2 (A-C): when compared with PBS, mean arterial pressure, heart rate, and pressure rate index, were similar between all experimental groups at all time points (2-way ANOVA, $P>0.05$).

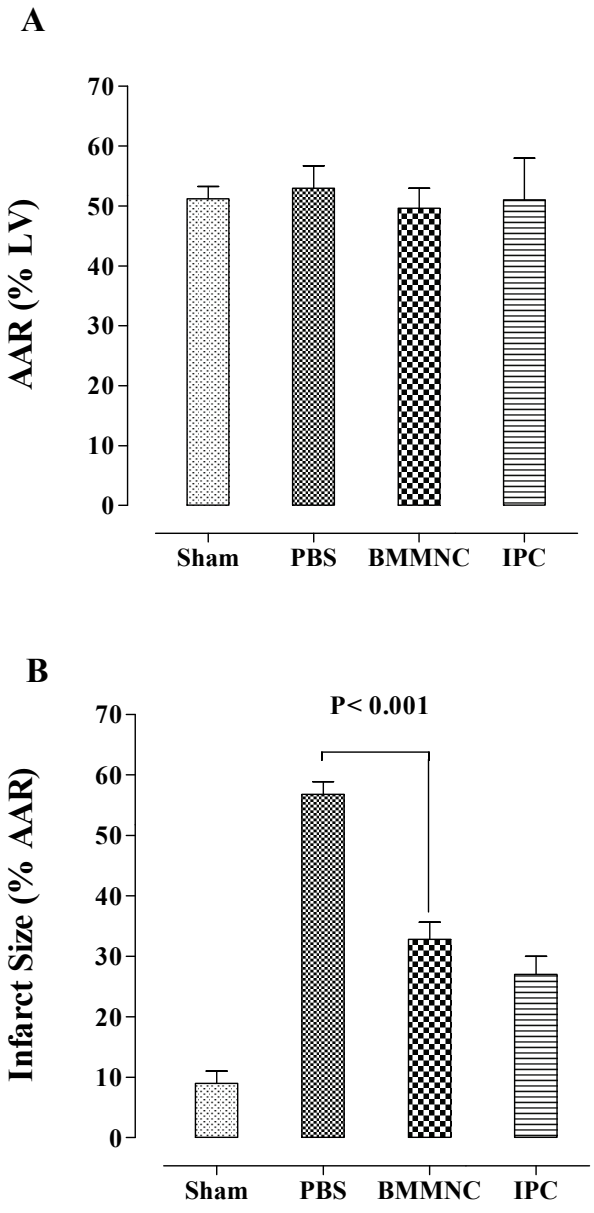


Figure 3.2.2.1 BMMNC therapy and infarct size following 25min LAD ischaemia and 2 h reperfusion (I/R-2h) (A) Left ventricular area at risk (AAR) from I/R-2h was similar between all groups. (**B**) When compared to PBS (control), infarct size (% AAR) was significantly attenuated by a single IV bolus of 10 million BMMNC upon reperfusion (1-way ANOVA and Dunnett's post test, P<0.001, n=10). Infarct size comparison between BMMNC and IPC was similar. Sham group underwent the same surgical procedure but no I/R-2h.

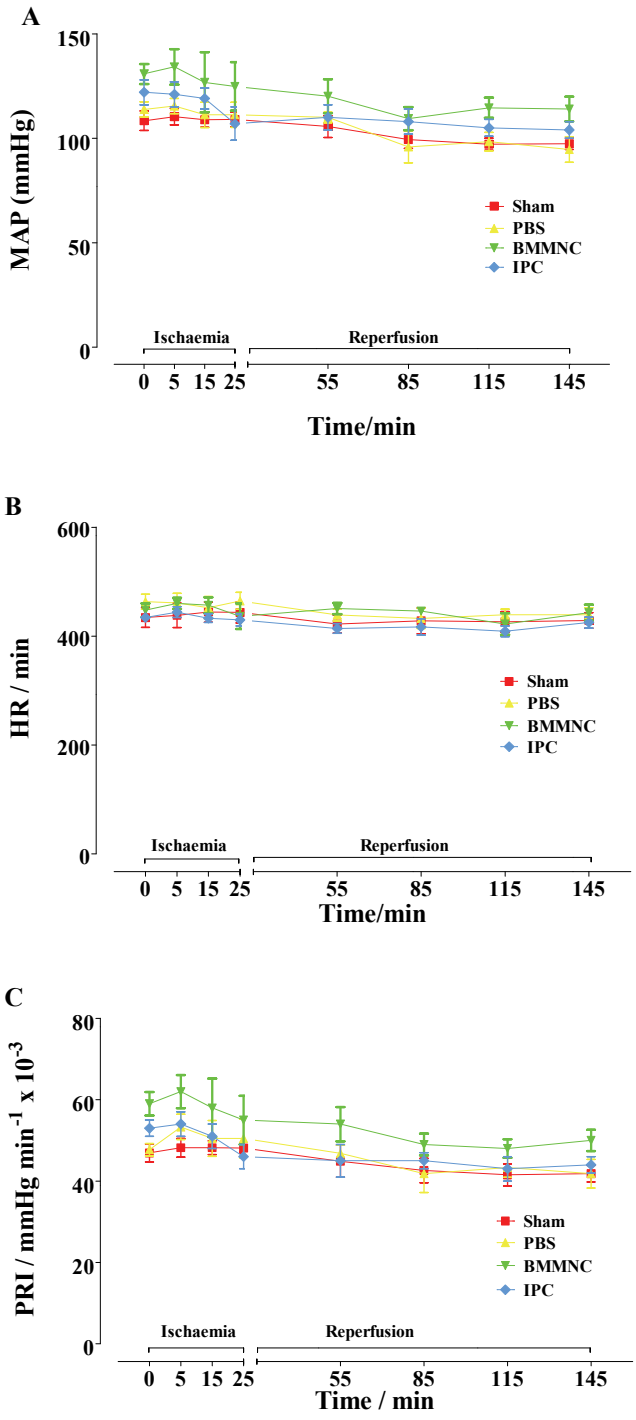


Figure 3.2.2.2 Haemodynamic variability during 25min LAD ischaemia and 2 h reperfusion. When compared with PBS (control) alterations in (**A**) mean arterial pressure (MAP), (B) heart rate (HR), and (C) pressure rate index (PRI) were similar between all experimental groups (2-way ANOVA, $P > 0.05$).

3.3 Intravenous BMMNC Therapy upon Reperfusion and Left Ventricular Function

3.3.1 Methods

Assessment of LV function in Wistar male rats was by transthoracic 2-D echocardiography and LV pressure transduction catheter as described in chapter 2. Assessment of LV function in animals was pre and post 25 min of left anterior descending coronary artery (LAD) occlusion and reperfusion for 7 days (I/R-7D). A delay of 7 days before assessment of LV function is to allow for the resolution of post-ischaemic ventricular stunning (Braunwald *et al.,* 1982) and optimisation of the echocardiographical image quality following surgical thoracotomy. Following 25 min LAD occlusion, animals received either a single bolus IV of 10 million BMMNC or PBS (vehicle), at the onset of reperfusion. Approximately, 1 h following the onset of reperfusion, the thoracotomy wound was closed and the anaesthetic discontinued. I carried out all the surgical steps and following I/R-7D the LV functional data acquisition and data analysis (2-D echocardiography plus LV pressure transduction catheter) was by blinded observers. The experimental groups were as shown in Table 3.3.1.1.

Table 3.3.1.1 Experimental Design: BMMNC therapy and LV function following 25min LAD ischaemia and 7 days reperfusion. Wistar male rats underwent either 25 min LAD occlusion and 7 days of LAD reperfusion (I/R-7D) or no LAD occlusion i.e. sham procedure. The n value is the number of animals in each group.

Group	**N**	**Reperfusion Protocol**	**IV Injection upon Reperfusion**
Sham	10	No I/R-7D	0.5 ml PBS
PBS	10	I/R-7D	0.5 ml PBS
BMMNC	10	I/R-7D	10×10^6 BMMNC (0.5 ml PBS)

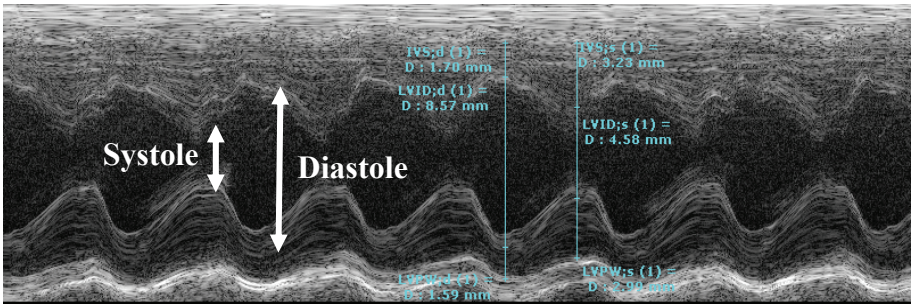
3.3.2 Results

In Figure 3.3.2.1, representative images of transthoracic 2-D echo used for the assessments of LV function show comparative M-mode images for sham, PBS and BMMNC animals. In Figure 3.3.2.2, when compared with BMMNC, both left ventricular ejection fraction (LVEF) and left ventricular area of contraction (FAC) were significantly attenuated for the PBS (vehicle control) group: LVEF (BMMNC 71 ± 3 % *vs.* PBS 48 ± 4 %, n = 10, $P < 0.0001$) and FAC (BMMNC 47 ± 2 % *vs.* PBS 36 ± 3 %, n = 10, $P < 0.01$). There were no significant differences in both the LVEF and FAC when comparing BMMNC with the sham group.

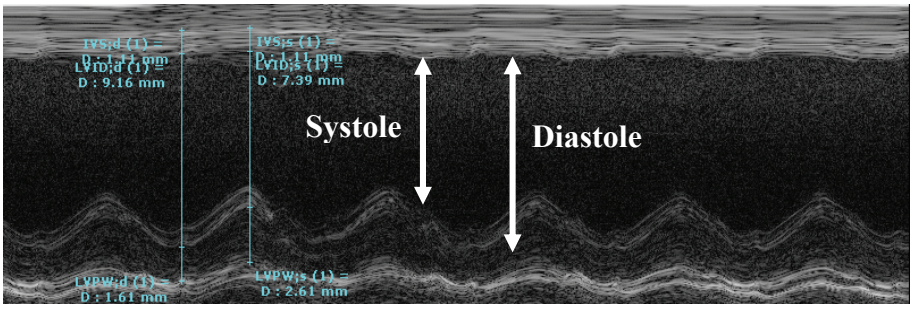
In Figures 3.3.2.3 and 3.3.2.4, the LV pressure transduction catheter derived parameters used to assess LV systolic function and diastolic function. Owing to technical difficulties, it was not possible to catheterize the LV in three animals. The LV pressure transduction catheter derived parameters used to assess systolic function were peak rate of increase of LV pressure (dP/dtmax) and contractility index. Both dP/dtmax and the contractility index were significantly reduced in the PBS treated group, as compared with the BMMNC group: dP/dtmax (BMMNC 10 x 10^3 $mmHgs^{-1}$ ± 0.4 % *vs.* PBS 8 x 10^3 $mmHgs^{-1}$ ± 1 % n = 7, $P < 0.05$); Contractility Index (BMMNC 162 ± 10 *vs.* PBS 113 ± 3, n = 7, $P < 0.001$). These results are concordant with the echocardiography derived LVEF and FAC.

Left ventricular diastolic dysfunction was demonstrated by an increase in left ventricular end-diastolic pressure (LVEDP), a decrease in the peak rate of decline in left ventricular pressure (-dP/dtmin), and an increase in the time constant of left ventricular pressure fall (tau). When compared with PBS, LV compliance was significantly higher for BMMNC treated group; LVEDP (BMMNC 4 ± 1 mmHg *vs.* PBS 16 ± 3 mmHg, $P < 0.01$); dP/dtmin (BMMNC -9 x 10^3 ± 0.5 $mmHgs^{-1}$ *vs.* PBS -7 x 10^3 ± 1 $mmHgs^{-1}$, $P < 0.01$); τ (BMMNC 10.2 x 10^{-3} ± 0.5 *vs.* PBS 12.3 x 10^{-3} ± 1, $P< 0.05$). These data suggest that the IV administration of BMMNC, upon reperfusion, preserved both systolic and diastolic function.

Sham



PBS



BMMNCC

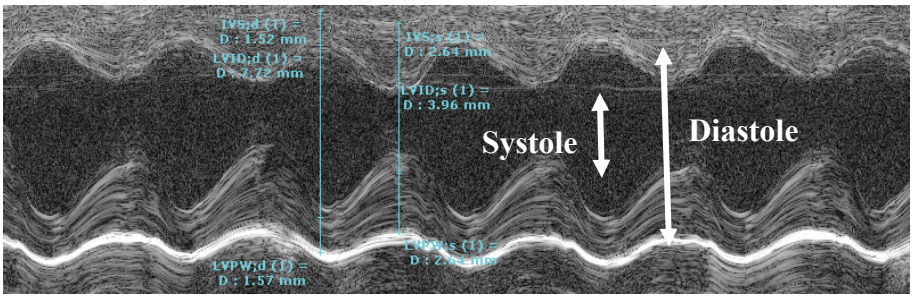


Figure 3.3.2.1 Representative transthoracic 2-D echo M-mode imaging. Assessment of LV function was following 25 min LAD ischaemia and reperfusion for 7 days (I/R-7D). When compared with PBS, LV function was significantly higher in animals treated with a single IV bolus of 10 million BMMNC upon reperfusion. The sham group underwent the same surgical procedure but no I/R-7D.

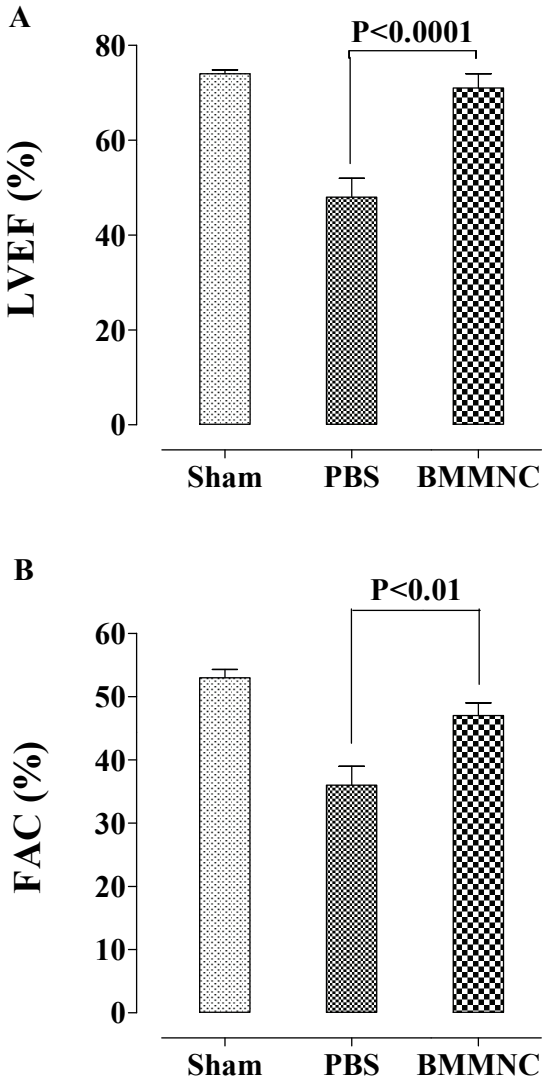


Figure 3.3.2.2 Transthoracic echocardiography following 25min LAD ischaemia and reperfusion for 7 days (I/R-7D). (**A**) When compared to PBS, the LV ejection fraction (LVEF) was significantly higher in animals treated by a single IV bolus of BMMNC (1-way ANOVA and Dunnett's post, P<0.0001, n=10). (**B**) When compared to PBS, LV fractional area of contraction (FAC) was also significantly higher for BMMNC (1-way ANOVA and Dunnett's post test, P<0.01, n=10).

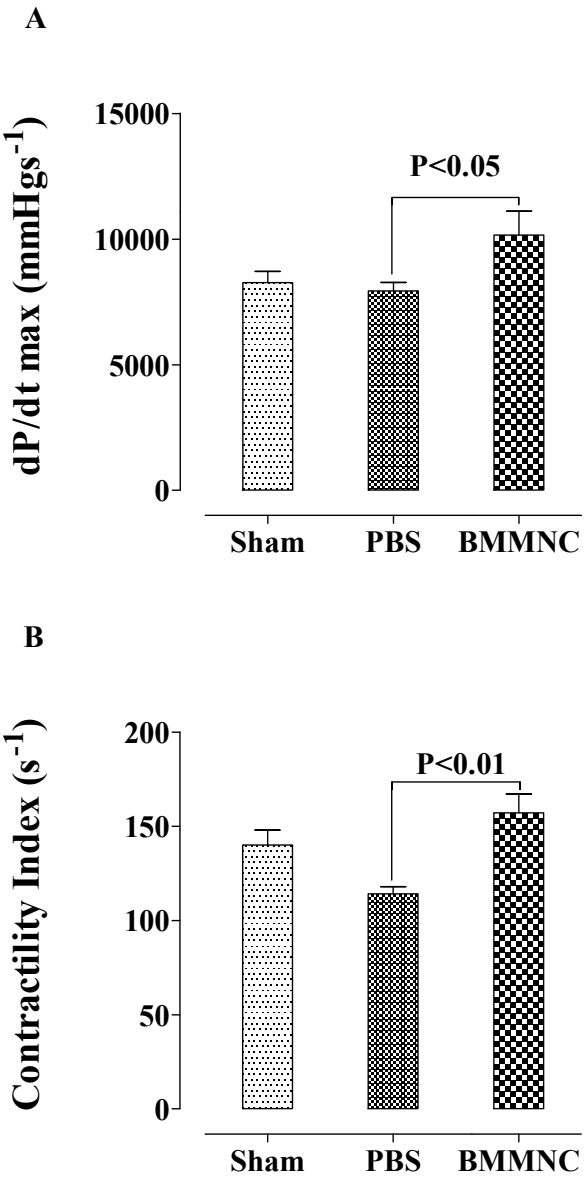


Figure 3.3.2.3 Left intraventricular pressure catheterisation following 25min LAD ischaemia and reperfusion for 7 days (I/R-7D). (A)When compared with PBS, rate of increase in systolic intraventricular pressure (dP/dt_{max}) was significantly higher in animals treated by a single IV bolus of 10 million BMMNC upon reperfusion (1-way ANOVA and Dunnett's post test, $P<0.05$, n=7). (B) When compared with PBS, LV contractility index was also significantly higher for BMMNC animals (1-way ANOVA and Dunnett's post test, $P<0.01$, n=7).

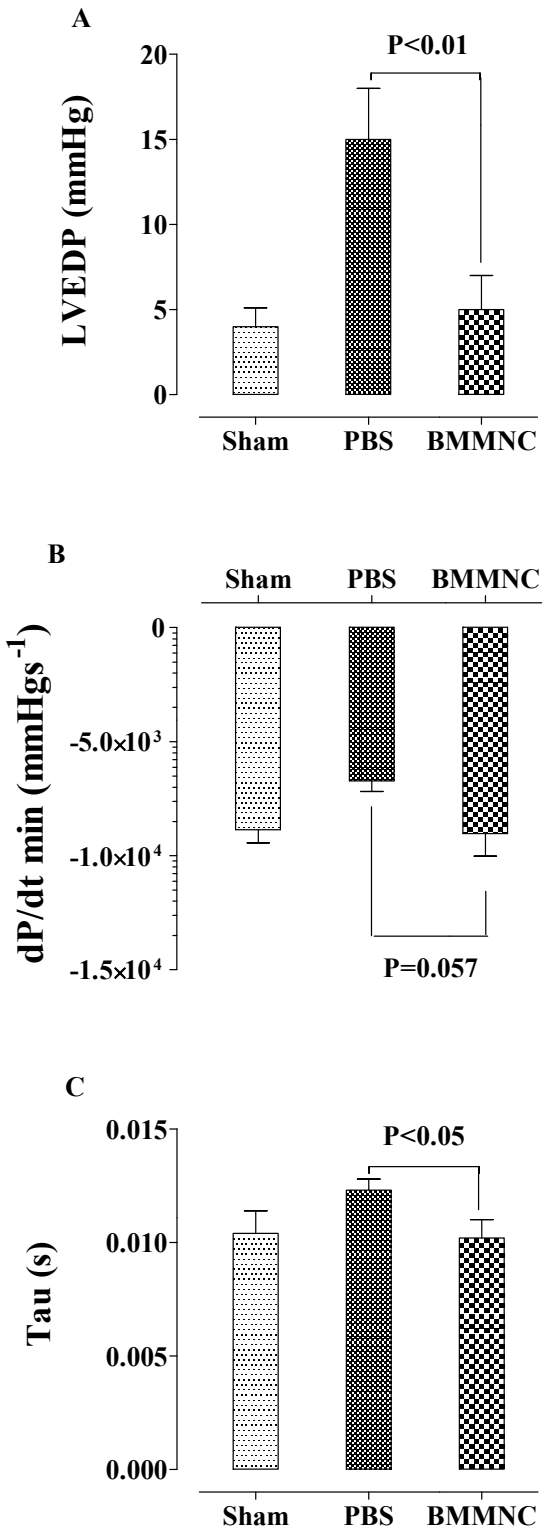


Figure 3.3.2.4 LV pressure catheterization following 25min LAD ischaemia and reperfusion for 7 days (I/R-7D). (A) When compared with PBS, LV end diastolic pressure (LVEDP) was significantly lower in animals treated by a single IV bolus of 10 million BMMNC, upon reperfusion (1-way ANOVA and Dunnett's post hoc test, P<0.01). (B) When compared with PBS, decrease in diastolic left ventricular pressure (dP/dt_{min}) was also lower for BMMNC but not statistically significance (1-way ANOVA and Dunnett's post hoc test, P=0.057). (C) When compared with PBS, LV diastolic time constant (Tau) was significantly lower for BMMNC (1-way ANOVA and Dunnett's post hoc test, P<0.05).

3.4 Intravenous BMMNC Therapy upon Reperfusion and Cardiac Fibrosis

3.4.1 Methods

A qualitative assessment of the volume of extracellular collagen deposited following regional I/R was by picrosirius red staining of myocardial cryosections. Cryosections were made of hearts, acquired from Wistar male rats that had previously been subjected to 25min LAD occlusion and 7 days reperfusion for assessment cardiac function in the previous study. Thus, donor BMMNC from syngeneic Wistar male rats were injected by an IV bolus, at the onset of reperfusion. After reperfusion had continued for more than 1 hour, the chest was closed and animals were recovered from general anaesthesia and then returned to the animal facility. Following 21 days of reperfusion, i.e. 14 days after assesement of cardiac function, rats were anaesthetised using 5% isoflurane and then sacrificed by cervical dislocation. The hearts were then excised and perfused with 10ml of ice cold PBS, followed by 5ml ice cold 4% paraformaldehyde (Sigma) via an 18 Ch cannula inserted in the ascending aorta and then immersed for 30 min in 4% paraformaldehyde on ice. Next the hearts were washed with PBS and incubated with 30% sucrose in PBS solution at 4°C overnight. Fixed hearts were then cut transversely into two segments between the base and apex. These segments were embedded in OCT compound (VWR) using a polyethylene mould and frozen in liquid nitrogen cooled isopentane and stored at -80°C. Cryosections (15-25μm) were cut from each segment using

a rotary cryotome (Leica) and then placed on polysine-coated glass slides (VWR), were washed with PBS and dried at room temperature overnight. The sections were then immersed in fast blue (0.15% Fast Blue in magnesium borate) for 10 min at room temperature, washed with de-ionised water, and stained with 0.1% picrosirius red F3B (0.1 g Sirus red in 100 ml saturated aqueous picric acid) for 10 min at room temperature. The sections were then rinsed in de-ionised water for 5 times and then immersed in picric alcohol (20 ml, absolute alcohol; 70 ml de-ionised water; 10 ml saturated aqueous picric acid). Sections were then dehydrated through a methanol series and mounted in DPX (VWR) and examined by light microscopy.

3.4.2 Results

A qualitative assessment of the post AMI collagen deposition was by cryosections of hearts stained with picrosirius red. Stained cryosections examined by light microscopy (Figure 3.4.2.1). When compared to the sham operated hearts, treatment by PBS upon reperfusion caused an extensive degree of extracellular collagen deposition within the infarct zone. Further, we noted the post infarct LV remodeling process by way of thinning and expansion of the infarct zone, LV cavity dilation, and hypertrophy of the LV zones remote from the infarct. In contrast, hearts that had been subjected to ischaemia and reperfusion and treated with BMMNC upon reperfusion had a much reduced collagen deposition within the anterior LV and post infarct LV remodeling was much less.

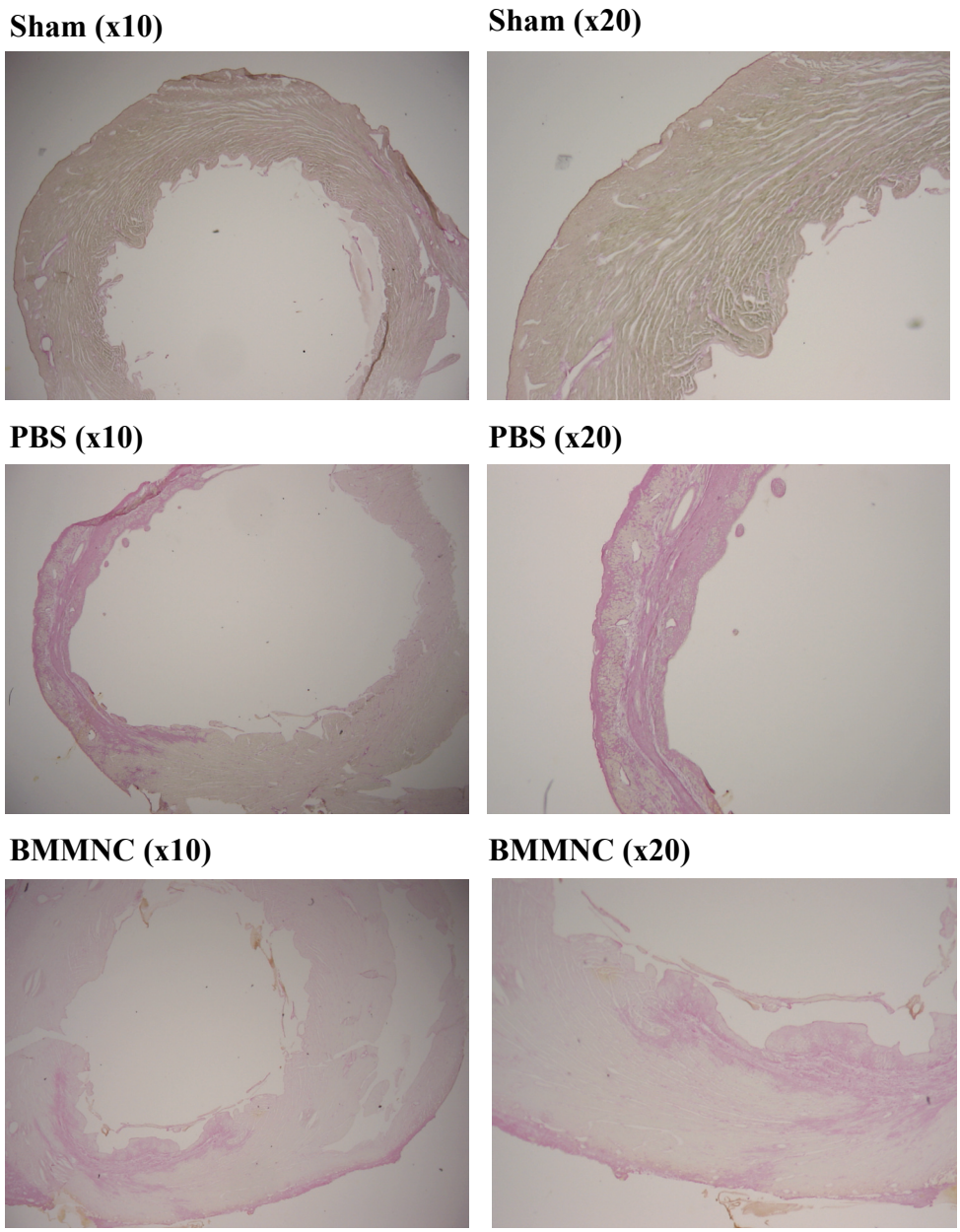


Figure 3.4.2.1 Representative picrosirius red stained cryosections of hearts from animals 21 days post 25min LAD ischaemia and reperfusion. When compared to PBS, a single IV bolus of 10million BMMNC upon reperfusion had lower collagen deposition. The sham underwent the same surgical procedure except for LAD ischaemia and reperfusion.

3.5 Discussion

I have presented data investigating whether systemic IV BMMNC therapy can protect the ischaemic myocardium from reperfusion injury. Firstly, IV BMMNC upon reperfusion achieved significant reductions in myocardial infarct size, which are comparable to the powerful and innate cardioprotective mechanism IPC. Secondly, in a recovery model of regional myocardial ischaemia and reperfusion (I/R-7D), IV BMMNC upon reperfusion also preserved systolic and diastolic function. Consistent with the preservation in cardiac function, I also report that IV BMMNC upon reperfusion prevented post infarct fibrosis and the associated adverse left ventricular remodeling.

Previously, studies had suggested that the plasticity of haematopoietic stem/progenitor cells might attenuate myocardial infarction by *de novo* myocardial regeneration of an infarcted myocardium. Thus, these studies sought to exploit the plasticity of engrafted haematopoietic stem/progenitor cell to undergo subsequent transdifferentiation. To encourage this event, investigators injected highly enriched haematopoietic stem/progenitor (CD34+/C-kit+) cells into non-reperfusing ischaemic myocardium (Kocher *et al.,* 2001; Orlic *et al.,* 2001). The landmark study by Orlic *et al.,* demonstrated that highly enriched syngeneic murine BMMNC (c-kit+/lin-) transdifferentiated to form a neo-vascularised and functional myocardium. Stem cell injection into the infarct border zone was followed by stem cell recruitment into the necrotic region for putative

regeneration of the transmural murine infarct, which was reported to be around 68%. This infarct regeneration was associated with significant improvements in the systolic (~40%) and diastolic function (~36%). Interestingly, the same group also reported in a separate study that injections of granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF) increased circulating haematopoietic stem/progenitor cells (c-kit + lineage -), which had the effect of significantly decreasing subsequent myocardial infarction (40%), LV dilation (26%), and diastolic stress (70%). More interestingly, Kocher *et al.*, demonstrated that IV injection of $2x10^6$ G-CSF mobilized *ex vivo* human BMMNCs were cardioprotective when injected 48 h following LAD ligation in a rat model of AMI. The donor human BMMNCs, that had been enriched for CD34+c-kit+ (>98% purity), were shown to immediately home into the infarct zone to subsequently stimulate neoangiogenesis, attenuate cardiomyocyte apoptosis, reduce LV collagen deposition, and improve cardiac function. I have presented data from experiments that did not use an enriched population of HSCs to protect against reperfusion injury of the ischaemic heart. Further, cell injection was by a single IV bolus at the onset of the reperfusing ischaemic myocardium i.e. a potentially hostile environment. These data suggest that the injected donor BMMNC could attenuate reperfusion-induced injury by a mechanism that was not entirely dependent upon donor cell plasticity. The data also suggest that pathophysiology for the reperfusion injury might be considered

separate from that of the injury due to ischaemia alone, as previously suggested (Hearse 1977; Vanden Hoek *et al.,* 1996).

The pathophysiology suggests that myocardial ischaemia and reperfusion injury is associated with two distinct modes of cell death, which are necrosis and apoptosis (Gottlieb *et al.,* 1999). Necrosis is an energy independent or passive process where lack of ATP decreases Na^+/K^+/ATPase ionic pump activity, which leads to accumulation of intra-cellular Na^+ ions, massive cell swelling, and eventual loss of membrane integrity. The consequential cell membrane disruption releases intra-cellular proteins and explains the acute inflammation reaction that is associated with tissue necrosis. Apoptosis, on the other hand, is a controlled energy dependent or active process which is observed to cause and organized degradation of nuclear and cytosolic contents, notably chromatin condensation, DNA fragmentation and apoptotic body formation. There is no cell membrane disruption and no acute inflammatory reaction (Fiers *et al.,* 1999). It would therefore not be unreasonable to suggest that necrosis is the predominant mode of cell death during ischaemia when ATP levels are diminished (Kajstura *et al.,* 1996) and that apoptosis is the predominant mode of cell death during reperfusion where ATP levels are normalizing (Zhao *et al.,* 2000). This simplistic separation of necrotic-ischaemia and apoptotic-reperfusion is, however, controversial and a number of studies suggest that cellular attrition throughout ischaemia and reperfusion is entirely by necrosis (Buja *et al.,* 1998; Ohno *et al.,* 1998; Taimor *et al.,*

1999). Most studies, however, suggest that bothe necrosis and apoptosis occur during ischaemia and the onset of reperfusion causes apoptosis to accelerate much faster than necrosis (Fliss *et al.,* 1996; Freude *et al.,* 2000; Gottlieb *et al.,* 1994; Holly *et al.,* 1999; Leist *et al.,* 1997; Mocanu *et al.,* 2000; Scarabelli *et al.,* 1999; Yaoita *et al.,* 1998; Zhao *et al.,* 2003; Zhao *et al.,* 2001). Thus, a potential therapeutic strategy to attenuate ischaemia and reperfusion injury would be to target the reperfusion component which is suggested to contribute approximately 50% of the total AMI (Yellon *et al.,* 2007). Hence, it has been shown that inhibition of apoptosis during reperfusion was associated with reduced infarct size and improved contractile function (Holly *et al.,* 1999; Mocanu *et al.,* 2000; Yaoita *et al.,* 1998; Zhao *et al.,* 2003). Intriguingly, the data I have presented suggests that the *hitherto* perceived role for stem cell therapy, i.e. only to regenerate dead myocardium might be additional to preventing its death in the first instance.

Chapter 4

Molecular Mechansisms Underlying Bone Marrow Mononuclear Cell Therapy and Attenuation of Regional Myocardial I/R Injury

4.1 Introduction

In the previous chapter, I have presented data suggesting that systemic IV BMMNC is cardioprotective when injected at the onset of reperfusion, in a rat model of regional myocardial transient ischaemia and reperfusion injury. The timing of cell injection suggests the injected cells may have migrated to the ischaemic myocardium along an SDF-1 gradient exposed to the peripheral circulation at the onset of reperfusion (Askari *et al.,* 2003). Notably, the clinical trials delivered autologous BMMNC after 24 h of reperfusion had elapsed, to prevent stem cell attrition in a hostile reperfusing myocardium (Chen *et al.,* 2004; Janssens *et al.,* 2006; Lunde *et al.,* 2006; Meyer *et al.,* 2006; Schachinger *et al.,* 2006). If indeed the injected BMMNC homing to the reperfusing myocardium do not survive, it is then conceivable that these cells might have a non-regenerative beneficial role for protecting the myocardium from I/R injury. Thus, in this chapter, my aim was to elucidate the molecular mechanisms that might be contributory to the putative non-regenerative benefits of the BMMNC therapy. Firstly, I investigated whether BMMNC attenuated cardiomyocyte

apoptosis or necrosis or both. Secondly, I investigated whether BMMNC secreted factors that might be contributing to the cardioprotection. Finally, I investigated any changes to the myocardial proteome brought about by BMMNC therapy.

4.2 Cardiomyocyte Apoptosis and Necrosis following BMMNC Therapy upon Reperfusion in Regional I/R Injury

4.2.1 Methods

BMMNC were isolated from *ex vivo* adult BM removed from the femurs and tibae of Wistar male rats, as described in chapter 2. Randomly selected Wistar male rats were subjected I/R-2h, as described. BMMNC delivery was by single IV injection at onset of reperfusion or at later time points during reperfusion. At the end of reperfusion, cardiomyocytes were isolated from the region of left ventricle (LV) that had been subjected to LAD ischaemia and reperfusion, the area at risk (ARR), as described in chapter 2 (Mitra *et al.,* 1985). Cardiomyocyte staining for apoptosis and necrosis, for FACS flow cytometric analysis, was as described in chapter 2.

Table 4.2.1.1 Experimental Design: BMMNC post reperfusion in a model of 25 min LAD ischaemia and 2 h reperfusion. Wistar male rats underwent either 25 min LAD occlusion and 2 h of LAD reperfusion (I/R-2h) or no LAD occlusion i.e. sham procedure. The time of either PBS or an IV bolus of BMMNC was from 0 min to 90 min following onset of reperfusion. n is the number of animals in each group.

Group	n	Protocol	IV Injectate	Time of Injection Post Reperfusion (min)
Sham	**5**	**No I/R-2h**	**0.5 ml PBS**	**0**
PBS	**5**	**I/R-2h**	**0.5 ml PBS**	**0**
0	**5**	**I/R-2h**	**10 x 10^6 BMMNC**	**0**
15	**5**	**I/R-2h**	**10 x 10^6 BMMNC**	**15**
30	**5**	**I/R-2h**	**10 x 10^6 BMMNC**	**30**
60	**4**	**I/R-2h**	**10 x 10^6 BMMNC**	**60**
90	**5**	**I/R-2h**	**10 x 10^6 BMMNC**	**90**

4.2.2 Results

In Figure 4.2.2.1(A), I have shown that the AAR (% LV), was similar between PBS (control) and treatment groups. In Figure 4.2.2.3, I have shown that the mean arterial pressure, heart rate and pressure rate index were not different between groups at all time points (2-way ANOVA, $P>0.05$). In figure 4.2.2.2, when compared to the PBS control group the AAR cardiomyocyte apoptosis was significantly reduced when BMMNC were injected within the first 30 min of reperfusion (0 min, 14 ± 3%; 15min, 15 ± 2%; 30 min, 11 ± 1%; versus PBS 28 ± 3%; $P<0.001$). This was concordant with the AAR cardiomyocytes expressing activated Caspase 9; i.e. when compared to PBS, Caspase 9 activation was significantly lower when BMMNC were injected within the first 30 min of reperfusion (0 min, 13 ± 3%; 15min, 16 ± 1%; 30 min, 16 ± 2%; versus PBS 31 ± 4%; 1-way ANOVA, Dunnett's post test $P<0.05$). In figure 4.2.2.1(B), when compared to the control group (PBS), cardiomyocyte necrosis was also significantly reduced when BMMNC were injected within the first 30 min of reperfusion (0 min, 5 ± 1%; 15 min, 8 ± 2%; 30 min, 8 ± 2%; versus PBS 16 ± 1%; 1-way ANOVA, Dunnett's post test $P<0.05$). Later injections of BMMNC, i.e. at 60 min and 90 min post onset of reperfusion, also attenuated cardiomyocyte apoptosis and necrosis upon injection, but these reductions did not reach statistical significance.

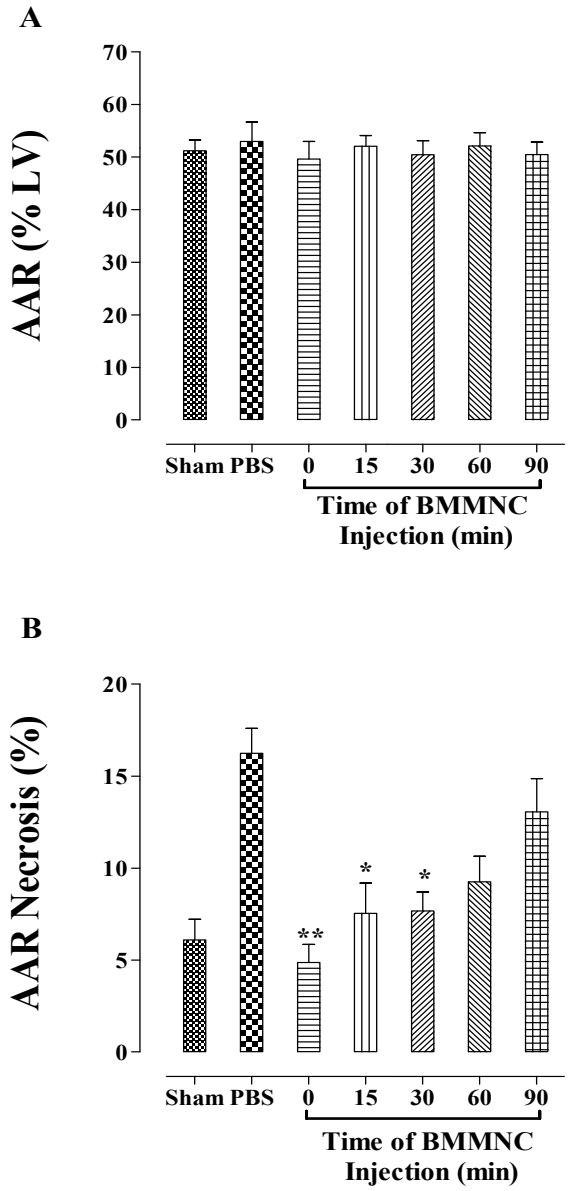


Figure 4.2.2.1 Area at risk (AAR) cardiomycyte necrosis detected by FACS flow cytometry (A) The AAR of the left ventricle (LV) subjected to 25 min LAD ischaemia and 2 h LAD reperfusion and expressed as % LV was similar between all groups (1-way ANOVA, $P>0.05$). **(B)** Compared to PBS, a single IV bolus of 10 million BMMNC at either 0 min, 15 min, or 30 min after the onset of reperfusion significantly reduced necrosis of cardiomyocytes isolated from the AAR (1-way ANOVA and Dunnett's post hoc test (** $P<0.01$, * $P<0.05$).

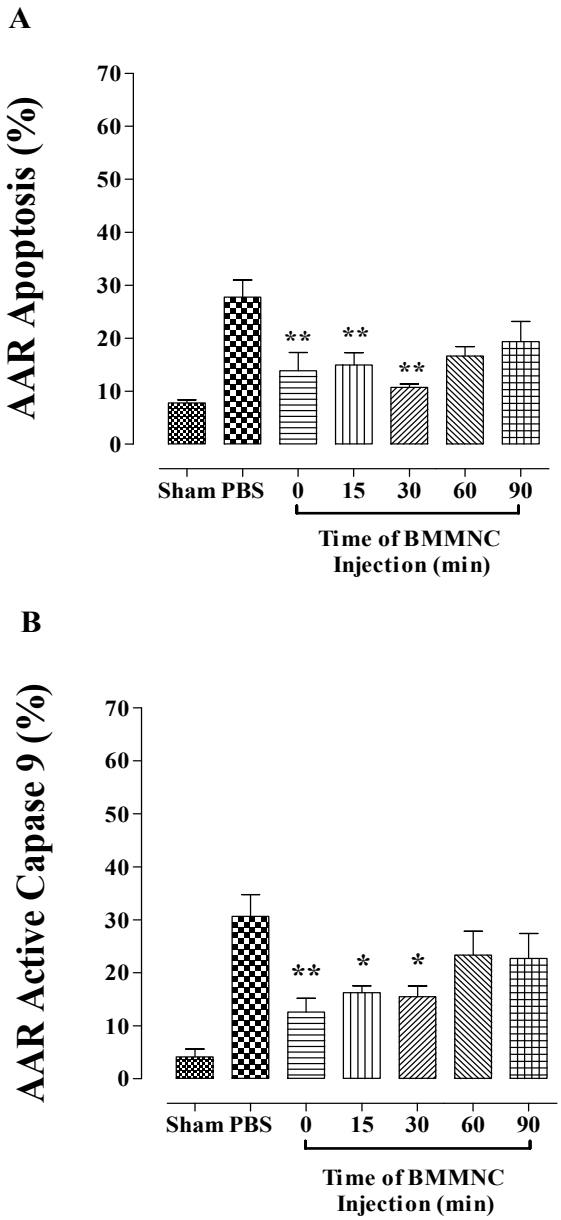


Figure 4.2.2.2 Area at risk (AAR) cardiomycyte apoptosis and caspase 9 activation detected by FACS flow cytometry (A) compared to PBS, 10 million BMMNC at 0 min, 15 min, or 30 min after the onset of reperfusion significantly reduced the number of apoptotic cardiomyocytes (1-way ANOVA and Dunnett's post hoc test, ** P<0.01).**(B)** Compared to PBS, BMMNC at 0 min, 15 min, or 30 min at reperfusion significantly reduced the number of cardiomyocytes with activated Caspase 9 (1-way ANOVA and Dunnett's post hoc test, ** P<0.01 and *P<0.05).

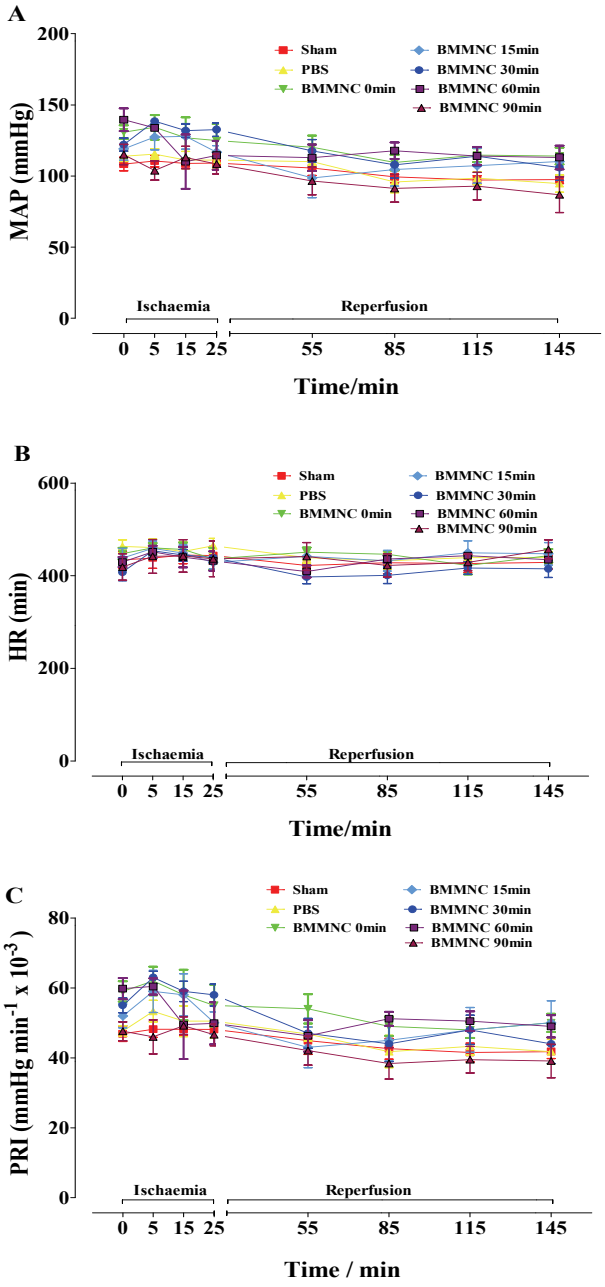


Figure 4.2.2.3 The haemodynamic parameters of animals subjected to 25 min LAD occlusion and 2 h LAD reperfusion were similar between all groups (**A**) Mean arterial pressure mmHg. (**B**) Heart rate (beats per minute). (**C**) Pressure rate index (PRI) the product of mean arterial pressure and heart rate (2-way ANOVA, $P > 0.05$).

4.3 Removal of Cells from BMMNC Therapy did Not Eliminate Cardioprotection

4.3.1Methods

BMMNC were isolated from *ex vivo* adult BM aspirated from femurs and tibae of Wistar male rats, as previously described. Extracellular factors as secreted by BMMNC were collected by incubating the cells in PBS and the resulting BMS was obtained in various concentrations by incubating increasing numbers of BMMNC in 1ml PBS: 10, 20, 50 or 100 million cells. The time of incubation was varied at either 2 h or 24 h in a cell culture incubator (5% CO_2 and humidified air at 37°C; CB150, USA). Post incubation, cells were separated from the supernatant by centrifugation (13000 rpm) followed by filtration (0.2 μm sterile syringe filter, Corning Life Sciences, USA). The supernatant was used immediately after preparation. Animals that were randomly selected to receive BMS were administered 0.5 ml BMS as a single IV bolus, at the onset of LAD reperfusion. At the end of 2 h reperfusion, the region of the left ventricle subjected to LAD ischaemia and reperfusion was analyzed for infarct size by observers blinded to each treatment group.

Table 4.3.1.1 Experimental Design: BMMNC derived supernatant over 2 h (BMS-2h) upon reperfusion in a model of 25 min LAD ischaemia and 2 h reperfusion. Wistar male rats underwent either 25 min LAD occlusion and 2 h of LAD reperfusion (I/R-2h) or no LAD occlusion i.e. sham procedure. BMS-2h obtained by increasing numbers of BMMNC over an incubation time of 2 h after which BMMNC were removed from the supernatant. An IV bolus of either PBS or BMS-2h was at the onset of reperfusion. n is the number of animals in each group.

Group	N	Protocol	IV Injectate	Number of BMMNC used to obtain BMS-2 h
0	3	I/R-2h	BMS-2h (0)	0
10	3	I/R-2h	BMS-2h (10)	10×10^6
20	3	I/R-2h	BMS-2h (20)	20×10^6
50	3	I/R-2h	BMS-2h (50)	50×10^6
100	3	I/R-2h	BMS-2h (100)	100×10^6

Table 4.3.1.2 Experimental Design: BMMNC derived supernatant over 24 h (BMS-24h) upon reperfusion in a model of 25 min LAD ischaemia and 2 h reperfusion. Wistar male rats underwent either 25 min LAD occlusion and 2 h of LAD reperfusion (I/R-2h) or no LAD occlusion i.e. sham procedure. BMS-24h obtained by increasing numbers of BMMNC over an incubation time of 24 h after which BMMNC were removed from the supernatant. An IV bolus of either PBS or BMS-24h was at the onset of reperfusion. n is the number of animals in each group.

Group	n	Protocol	IV Injectate	Number of BMMNC used to obtain BMS-24h
0	7	I/R-2h	BMS-24h (0)	0
10	3	I/R-2h	BMS-24h (10)	10×10^6
20	6	I/R-2h	BMS-24h (20)	20×10^6
50	5	I/R-2h	BMS-24h (50)	50×10^6
100	4	I/R-2h	BMS-24h (100)	100×10^6

4.3.2 Results

In Figure 4.3.2.1 (A), I have presented the AAR (% of LV) and this was similar between controls and treatment groups (1-way ANOVA, $P>0.05$). In Figure 4.3.2.3, I have presented the data for the haemodynamic parameters of mean arterial pressure, heart rate and pressure rate index. They were similar between all groups and at all time points (2-way ANOVA, $P>0.05$). In Figure 4.3.2.1 (B), I have presented the infarct size data and when compared to the control group (PBS), IV injection of BMS derived after 2 h of incubation did cause non-significant reductions in infarct size, despite increasing the number of BMMNC numbers used to obtain BMS.

In Figure 4.3.2.2 (B), in contrast, when compared to the PBS group, BMS derived after 24 h cell incubation caused greater reductions in the infarct size, which achieved statistical significance when BMS was obtained from at least 20 million BMMNC (PBS 57 ± 4 % versus BMS-20, 35 ± 3 %; 1-way ANOVA and Dunnett's post test, $P < 0.01$). Maximal attenuation of the LV infarct size was notable where BMS was from 50 million or more BMMNC. Thus, removing the cells from BMS did not eliminate cardioprotection; however, equivalent cardioprotection was only achieved when BMS had been obtained after a 24 h incubation with BMMNC that were double in number (or greater) to that required for cell therapy.

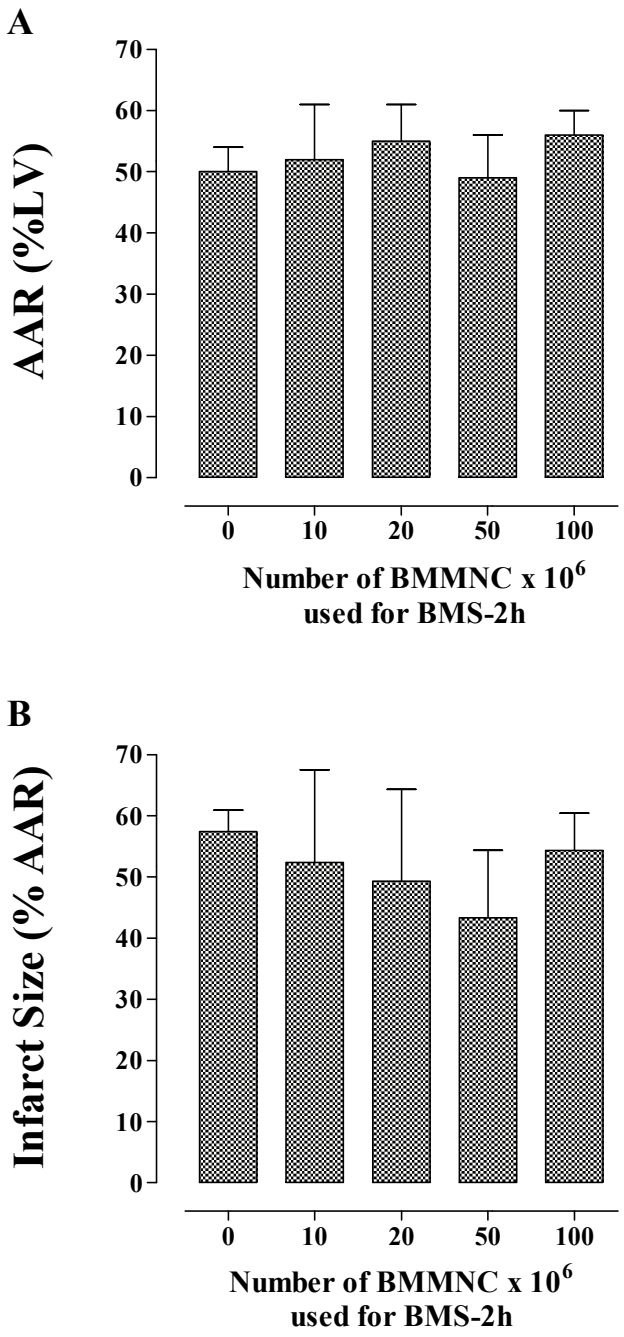


Figure 4.3.2.1 Infarct size and cell-free supernatant (BMS-2h) from BMMNC after 2 h incubation (A) The area at risk (AAR) from 25 min LAD ischaemia and 2 h LAD reperfusion, (% LV) was similar between all groups (1-way ANOVA, P>0.05). **(B)** When compared to the PBS, infarct size was non-significantly reduced by a single IV bolus of BMS-2h despite increasing the BMMMC number to obtain BMS-2h (1-way ANOVA, P>0.05).

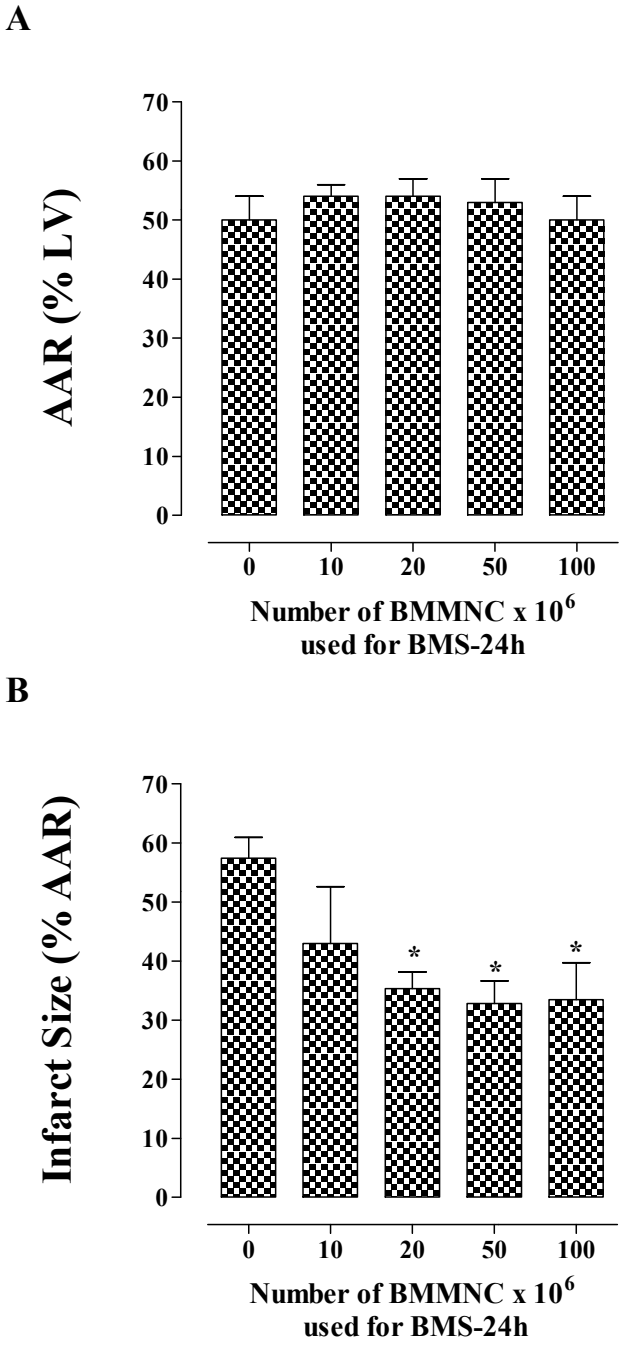


Figure 4.3.2.2 Infarct size and cell-free supernatant (BMS-24h) after 24 h incubation of BMMC (A) The area at risk (AAR) from 25 min LAD ischaemia and 2 h LAD reperfusion, expressed as % LV was similar between all groups (1-way ANOVA, P>0.05). **(B)**When compared to the PBS, infarct size was reduced by a single IV bolus of BMS-24h and this reduction was statistically significant for BMS-24h from 20 million or more BMMNC (1-way ANOVA and Dunnett's post test, *P < 0.01, n=6).

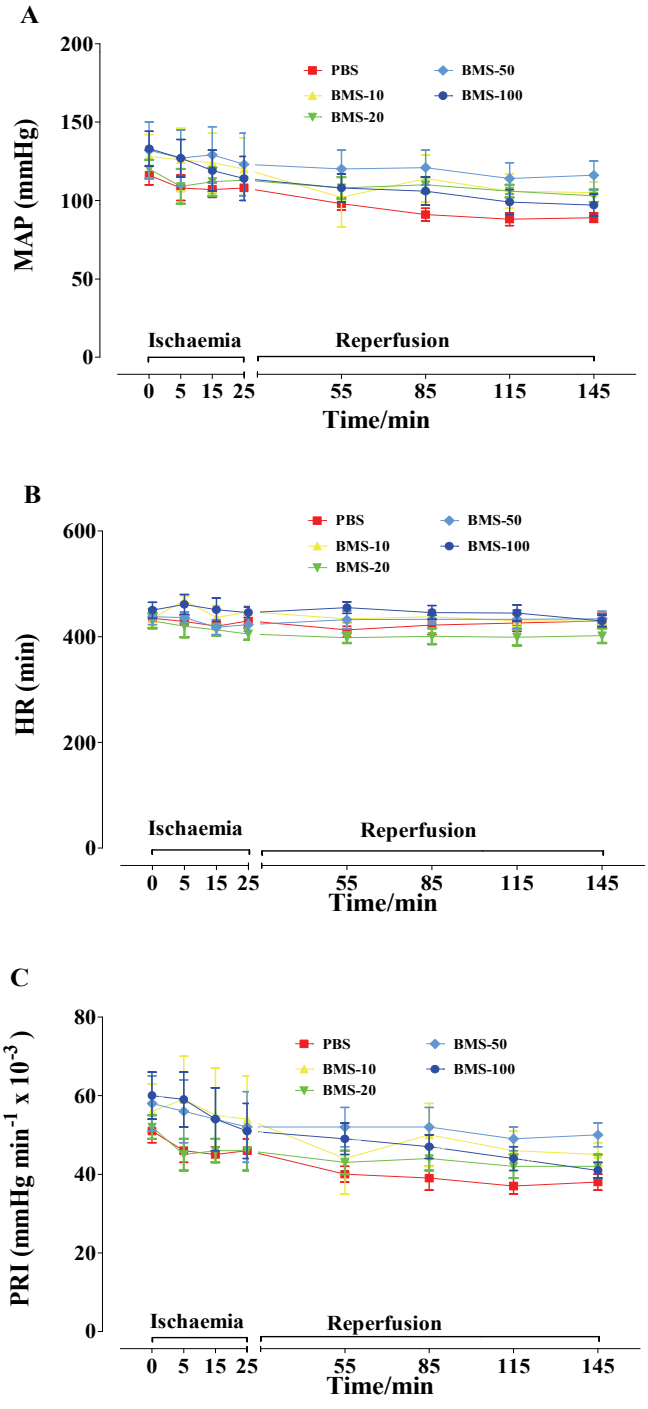


Figure 4.3.2.3 Haemodynamic variability and cell-free supernatant (BMS-24h) from BMMNC after 24 h incubation. When compared with PBS (control) alterations in (**A**) mean arterial pressure (MAP), (B) heart rate (HR), and (C) pressure rate index (PRI) were similar between all experimental groups (2-way ANOVA, $P > 0.05$).

4.4 Modulation of the Left Ventricular Proteome by BMMNC or BMS Therapy post Regional I/R

4.4.1 Methods

BMMNC were isolated from *ex vivo* adult BM aspirated from femurs and tibae of Wistar male rats, as previously described. Randomly selected Wistar male rats underwent 25 min LAD ischemia and 2 h of reperfusion. Animals received either 10 million BMMNC or BMS obtained from 50 million cells over 24 h. Administration was by a single IV bolus at the onset of reperfusion and control animals received PBS. Sham controls underwent the same surgical manipulation but not LAD ischaemia and reperfusion. At the end of reperfusion, the *ex vivo* left ventricular area at risk (AAR) was rapidly cooled by immersion in liquid nitrogen and stored in a freezer (-80°C) for subsequent proteomic analysis, as described in methods (chapter 2).

4.4.2 Results

Gel image analysis of triplicate 2-DE gels for each experimental group revealed 464 matched protein spots between the four experimental groups (Figure 4.4.2.1). When comparing the spot intensities of matched spots, 87 spots had differences between the experimental groups.When comparing protein expression of PBS controls, hearts treated with either BMMNC or BMS demonstrated significantly higher expression of a number of proteins which are shown in Table 4.4.2.2

Specifically, when compared to sham hearts, the expression of anti-apoptotic signal transduction protein 14-3-3 epsilon was 1.5 fold lower in PBS treated hearts. In contrast, both BMMNC and BMS treated hearts demonstrated a 1.5 fold higher expression of the anti-apoptotic signal transduction protein 14-3-3 epsilon when compared with PBS treated control hearts. When compared to sham hearts, the expression of the antioxidant peroxiredoxin-6 was 2.3 fold lower in PBS treated hearts. The expression of the other differentially expressed antioxidant catalase was similar, when comparing the PBS and sham treated hearts. The expression of both antioxidants catalase and peroxiredoxin-6 was at least two folds higher in the BMMNC treated hearts than in the PBS hearts, whilst only the expression of peroxiredoxin-6 was 1.6 fold higher for the BMS treated hearts than in the PBS hearts. Turning to the expression of heat shock protein (HSP), when compared to sham hearts, the expression of HSPs was lower in PBS treated hearts for both the low and the high molecular weight HSPs. Conversely, compared to the PBS control, BMMNC treated hearts had increased expression of HSP 20 (3.0 folds), alpha-B-crystallin (1.7 folds), HSP 72 (2.8 folds), TNF-Receptor Associated Protein-1 (2.3 folds), and Ischaemia Responsive Protein-94 (1.6). This trend of higher HSP expression was also demonstrated when comparing PBS controls with the BMS treated animals, however, this was noted for only three HSP, namely alpha-B-crystallin (1.5 folds), HSP 72 (2.1 folds), TNF, and Ischaemia Responsive Protein-94 (1.5).

There were also changes in the expression of cytosolic and mitochondrial bioenergetic proteins; when compared with the sham hearts, PBS treated hearts had lower expression of alpha-Enolase (2.3 folds). Conversely, on comparing the PBS control hearts, BMMNC hearts had higher expression of alpha-Enolase (1.6 folds) and Glyceraldehyde-3-Phosphate Dehydrogenase (2.3 folds). Similarly, comparing the BMS treated hearts also showed a trend for increased expression of glycolytic proteins by the higher expression of Glyceraldehyde-3-phosphate dehydrogenase (1.8 folds). The expression of fatty acid oxidation proteins was remarkably different to that noted for the glycolytic proteins. When compared with the sham-operated hearts, the PBS treated hearts' expression of acyl-co-A reductases and carboxylase was slightly higher. In sharp contrast, treatment with BMMNC caused the hearts to have a lower expression of acyl-co-A reductases and carboxylase. The trend for reduced expression of fatty acid acyl-co-A reductases and carboxylase expression was also apparent in the BMS treated hearts. There was attenuation of mitochondrial respiratory protein expression in the PBS treated hearts attenuated, when compared to the sham hearts: aconitase (3.4 folds) and voltage dependent anion channel protein-1 (1.5 fold). Whilst there was increased expression of these proteins in the both the BMMNC and the BMS treated hearts. Interestingly glutamate dehydrogenase expression was unchanged in PBS hearts but significantly decreased in both the BMMNC and the BMS treated hearts.

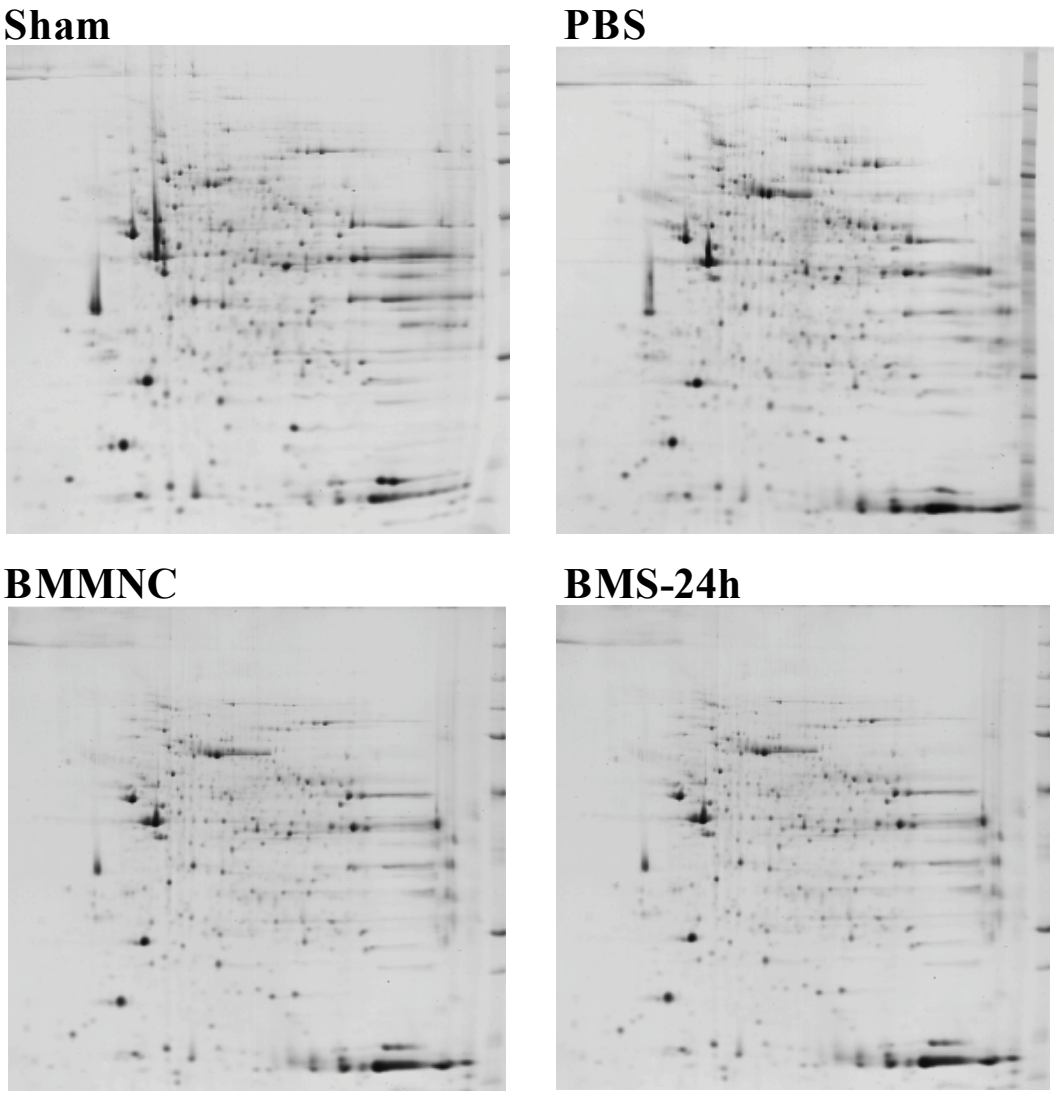


Figure 4.4.2.1 The 2-dimension gel electrophoresis images showing protein separation of protein homogenates from the left ventricular area at risk. Protein homogenates were derived from area at risk of hearts subjected to 25 min LAD ischemia followed by 2 h reperfusion or sham animals (no LAD ischaemia and reperfusion).

Protein	Role	PBS versus Sham (folds)	BMMNC versus PBS (folds)	BMS versus PBS (folds)	Mascot Score	Mr	pI	Peptides Matched	% Total Sequence Coverage
14-3-3 epsilon	Signaling	↓ (1.5)	↑ (1.5)	↑ (1.5)	489	29274	4.5	12	41
Peroxiredoxin-6	Antioxidant	↓ (2.3)	↑ (2.1)	↑ (1.6)	351	24672	6.5	5	27
Catalase	Antioxidant	↑ (1.2)	↑ (2.6)	↓ (1.1)	193	59931	7.15	4	8
HSP 20	HSP	↓ (13)	↑ (3)	↓ (1.3)	93	17551	6.05	3	19
Alpha B-crystallin	HSP	↓ (3.0)	↑ (1.7)	↑ (1.5)	207	20155	6.84	6	29
HSP 72	HSP	↓ (4.3)	↑ (2.8)	↑ (2.1)	261	71055	5.37	6	12
TNF-Receptor Associated Protein 1	HSP	↓ (2.7)	↑ (2.3)	↑ (1.3)	321	80639	6.56	7	12
Ischaemia Responsive Protein-94	HSP	↓ (1.6)	↑ (1.6)	↑ (1.5)	166	94795	5.13	4	5
Alpha-enolase	Glycolysis	↓ (2.3)	↑ (1.6)	1.0	717	47309	6.16	15	38
Glyceraldehyde 3-Phosphate Dehydrogenase	Glycolysis	1.0	↑ (2.3)	↑(1.8)	170	36098	8.43	4	11
Aconitase	Mitochondrial Respiratory	↓ (3.4)	↑ (4.7)	↑(4.3)	398	86162	7.87	12	17
Citrate synthase	Mitochondrial Respiratory	↑ (1.1)	↑ (2.3)	↓(1.7)	132	52176	8.53	5	11
Voltage Dependent Anion Channel Protein 1	Mitochondrial Respiratory	↓ (1.5)	↑ (2.7)	↑(1.8)	308	30720	8.63	5	19

Table 4.4.2.2 Differential protein expression as analyzed by proteomics of the left ventricular area at risk post 25 min LAD ischaemia and 2h reperfusion. The group comparisons were: (1) PBS versus sham; (2) BMMNC versus PBS; (3) Supernatant from 50 million BMMNC after 24 h (BMS) versus PBS. Protein spots with significant folds increase (↑) or decrease (↓) underwent analysis by mass spectroscopy to identify each protein by number of peptides matched, the total percentage of peptide sequence coverage, and the corresponding Mascot score. A Mascot score of greater than 30 suggests protein identification with a statistical significance of $P < 0.05$. Mr was the observed molecular weight (Daltons) and pI denotes isoelectric pH.

4.5 BMMNC Therapy and Cell Signaling

4.5.1 Methods

Having shown that both BMMNC and the BMMNC derived supernatant, (BMS) were equally cardioprotective, in a rat model of LAD regional ischaemia and myocardial reperfusion, I investigated the signaling pathways for BMMNC mediated cardioprotection. The increased expression of 14-3-3 epsilon by both BMMNC and BMS treated hearts suggested that PI3K/Akt survival kinase pathway might have a role in BMMNC mediated cardioprotection. Phosphorylation of BAD by Akt causes it to bind to 14-3-3 epsilon instead of the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Zha *et al.,* 1996). Thus, PI3K/Akt signaling was investigated.

BMMNC were isolated from *ex vivo* adult BM aspirated from femurs and tibae of Wistar male rats, as previously described. Infarct size was measured in randomly selected Wistar male rats that had been treated with the PI3K inhibitor LY294002 (0.3mg/ kg), by slow IV infusion 10 min prior to 25 min LAD ischaemia and 2 h reperfusion (Table 4.5.1).

In order to detect PI3K/Akt signaling, randomly selected Wistar male rats underwent 25 min LAD ischemia and only 30 min of reperfusion. Animals received 10 million BMMNC by single IV bolus at the onset of reperfusion and control animals received PBS. Sham controls underwent the same surgical manipulation but not LAD ischaemia and reperfusion (Tables 4.5.1.1 & 2). At the end of reperfusion, the left ventricular area at risk

(AAR) was rapidly cooled by immediate immersion in liquid nitrogen and storage in a deep freezer at -80°C for subsequent western blot analysis, as described in methods (chapter 2). Western blots of protein extracts from AAR protein homogenates were analyzed for phosphorylation of Akt, GSK-3β, p38 MAPK and NF-κB. Precise details of the western blotting techniques are given in Chapter 2.

Table 4.5.1.1 Experimental Design: Infarct size and pre-treatment with LY294002 and injection of IV BMMNC upon reperfusion in a model of 25 min LAD ischaemia and 2 h reperfusion (I/R-2h). Wistar male rats pretreatment with LY294002 (0.3mg/kg, IV) 10 min prior to the onset of either I/R-2h or no LAD occlusion i.e. sham procedure. Either PBS or a bolus of 10 million BMMNC was at onset of reperfusion. n is the number of animals in each group.

Group	n	Protocol	Pre-Ischaemia Injectate	Reperfusion Injectate
Sham	5	No I/R-2h	LY294002 (0.3 mg/ kg)	PBS (0.5 ml)
PBS	6	I/R-2h	LY294002 (0.3 mg/ kg)	PBS (0.5 ml)
BMMNC	6	I/R-2h	LY294002 (0.3 mg/ kg)	BMMNC (10×10^6)

Table 4.5.1.2 Experimental Design: Cell signaling analysis following pre-treatment with either LY294002 (0.3mg/kg) or dimethyl sulphoxide (DMSO) 10% in a model of 25 min LAD ischaemia and 30 min reperfusion (I/R-30min). Wistar male rats were pretreated with either DMSO 10% (vehicle) or LY294002 (0.3 mg/kg, IV) 10 min prior to the onset of either I/R-30min or sham procedure. Either PBS or a bolus of 10 million BMMNC were given upon reperfusion and n is the number of animals in each group.

Group	n	Protocol	Injectate Pre-Ischaemia	Injectate Upon Reperfusion
Sham	5	No I/R-30min	DMSO 10%	PBS
PBS	5	I/R-30min	DMSO 10%	PBS
LY294002 + PBS	6	I/R-30min	LY294002	PBS
BMMNC	3	I/R-30min	LY294002	BMMNC (10×10^6)
LY294002 + BMMNC	3	I/R-30min	LY294002	BMMNC (10×10^6)

4.5.2 Results

When compared with controls, pre-ischaemia treatment with LY294002 and regional myocardial ischaemia-reperfusion injury (I/R) caused a significant myocardial infarct; the injection of BMMNC upon reperfusion could not attenuate the size of infarction (Figure 4.5.2.1 B). There was no difference between groups when comparing the AAR and the haemodynamic parameters (Figures 4.5.2.1 A & 4.5.2.2).

When compared to the PBS controls, BMMNC injection upon reperfusion caused significant $Akt^{serine473}$ phosphorylation and pre-ischaemia treatment with LY294002 ameliorated BMMNC mediated $Akt^{serine473}$ phosphorylation (Figure 4.5.2.3). When compared to the PBS controls, BMMNC injection upon reperfusion caused significant GSK-$3\beta^{serine9}$ phosphorylation and pre-ischaemia treatment with LY294002 ameliorated BMMNC mediated GSK-$3\beta^{serine9}$ phosphorylation. Conversely, compared to the PBS controls, BMMNC injection upon reperfusion caused significant reductions in both the phosphorylation of p38-MAPK and nuclear translocation of NF-κB. Thus, pre-ischaemia treatment with LY294002 ameliorated BMMNC mediated inhibition of both p38-MAPK and NF-κB (Figure 4.5.2.4).

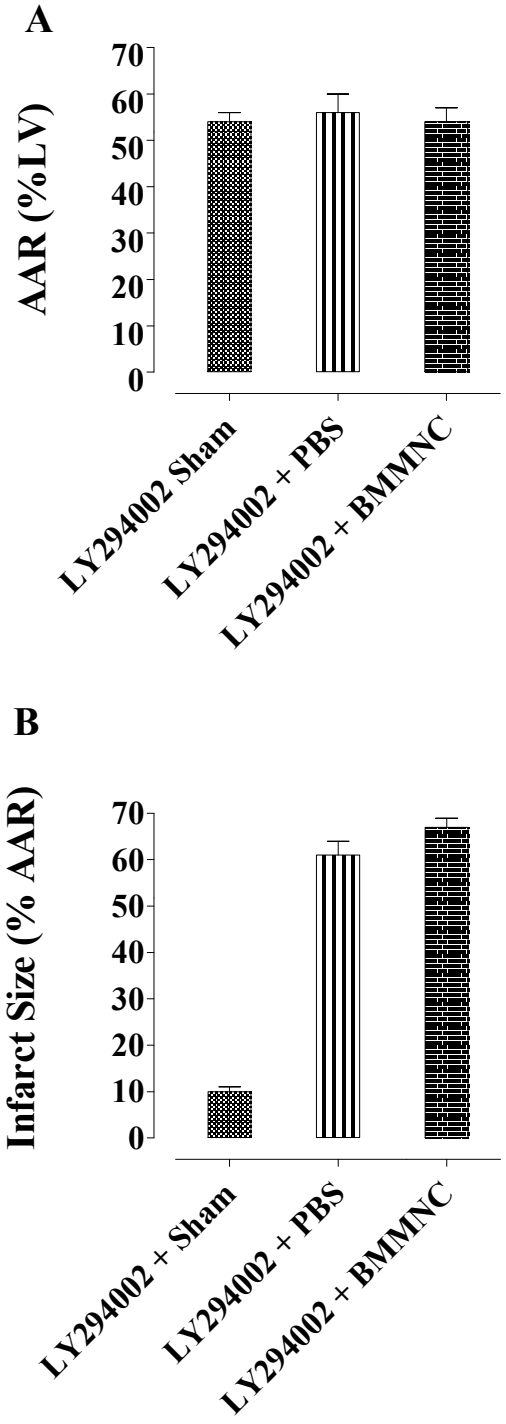


Figure 4.5.2.1 Infarct size and pre-treatment with LY294002 (A) The area at risk (AAR) from 25 min LAD ischaemia and 2 h LAD reperfusion was with LY294002 (0.3mg/kg). When compared to PBS, infarct size was similar to the group receiving a single IV bolus of 10 BMMNC upon reperfusion (1-way ANOVA and Dunnett's post test, $P>0.05$, $n=6$).

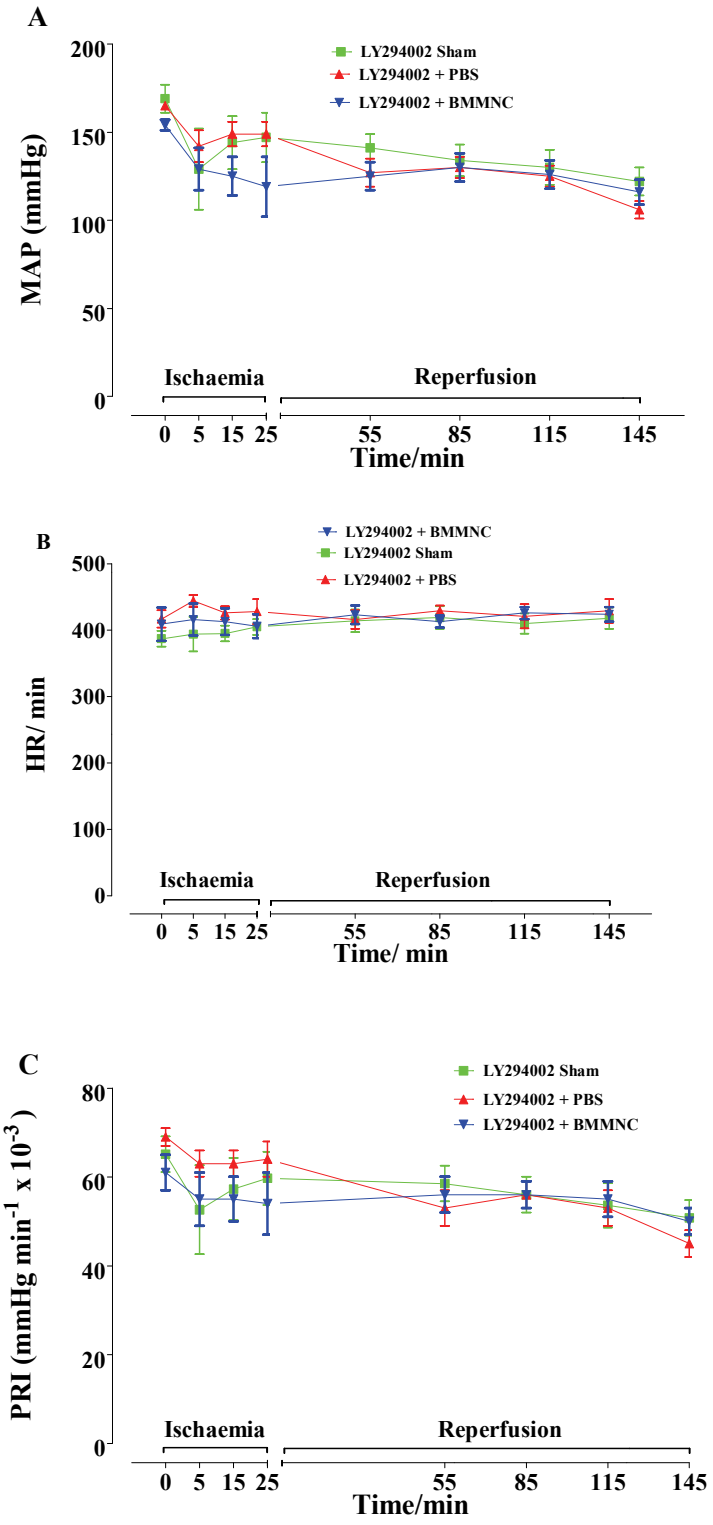


Figure 4.5.2.2 The haemodynamic parameters were similar between all experimental groups from baseline to the end of each experiment. (A) Mean arterial pressure (MAP); (B) Heart rate (HR); (C) Pressure rate index (PRI) (2-way ANOVA, $P > 0.05$).

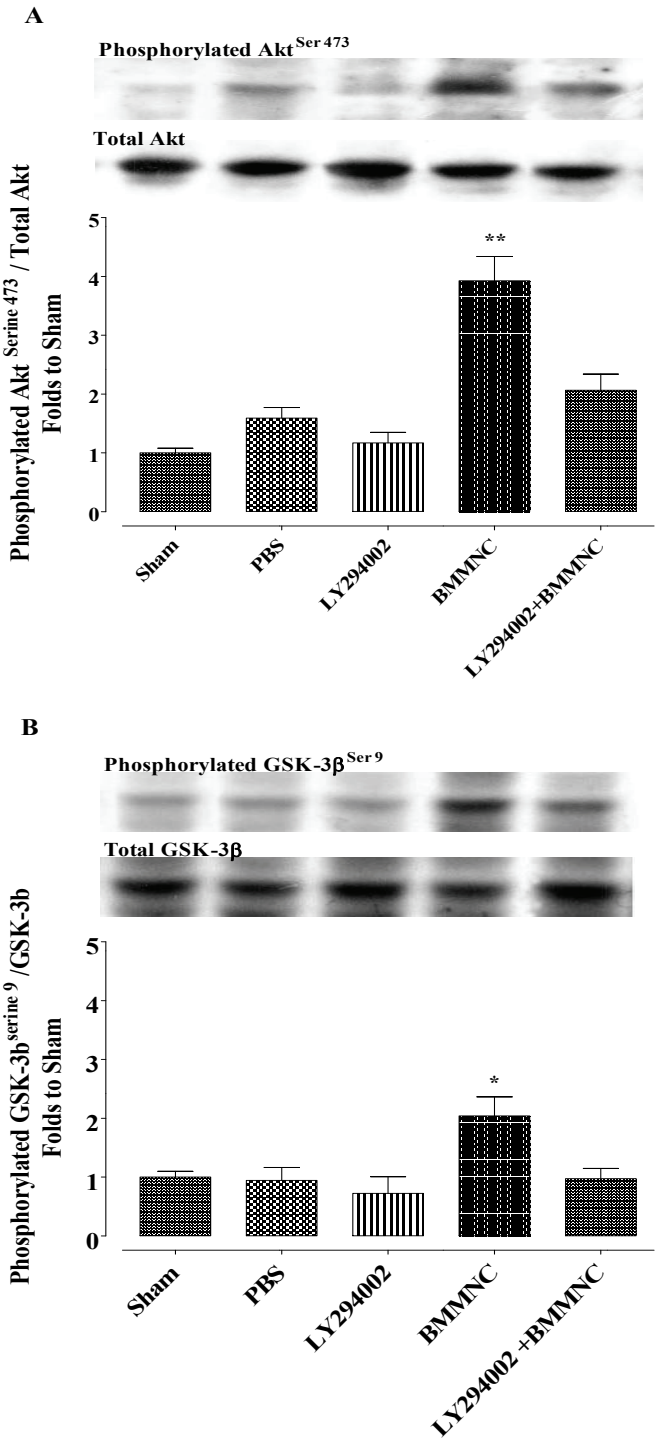


Figure 4.5.2.3 Western blots of left ventricular area at risk 30 min post LAD reperfusion to detect phosphorylated cytosolic Akt and glycogen synthase kinase-3β(GSK-3β). (A) When compared with phosphate buffered saline (PBS), an IV bolus of 10 million bone marrow mononuclear cells (BMMNC) upon reperfusion significantly augmented the phosphorylation of Akt at serine 473. Akt phosphorylation is significantly lower following pre-treatment with LY294002. **(B)** Compared to PBS, BMMNC upon reperfusion also significantly phosphorylated GSK-3β at serine 9, which was significantly lower following pre-treatment with LY294002. 1-way ANOVA and Dunnett's post test; **P<0.01 and *P< 0.05.

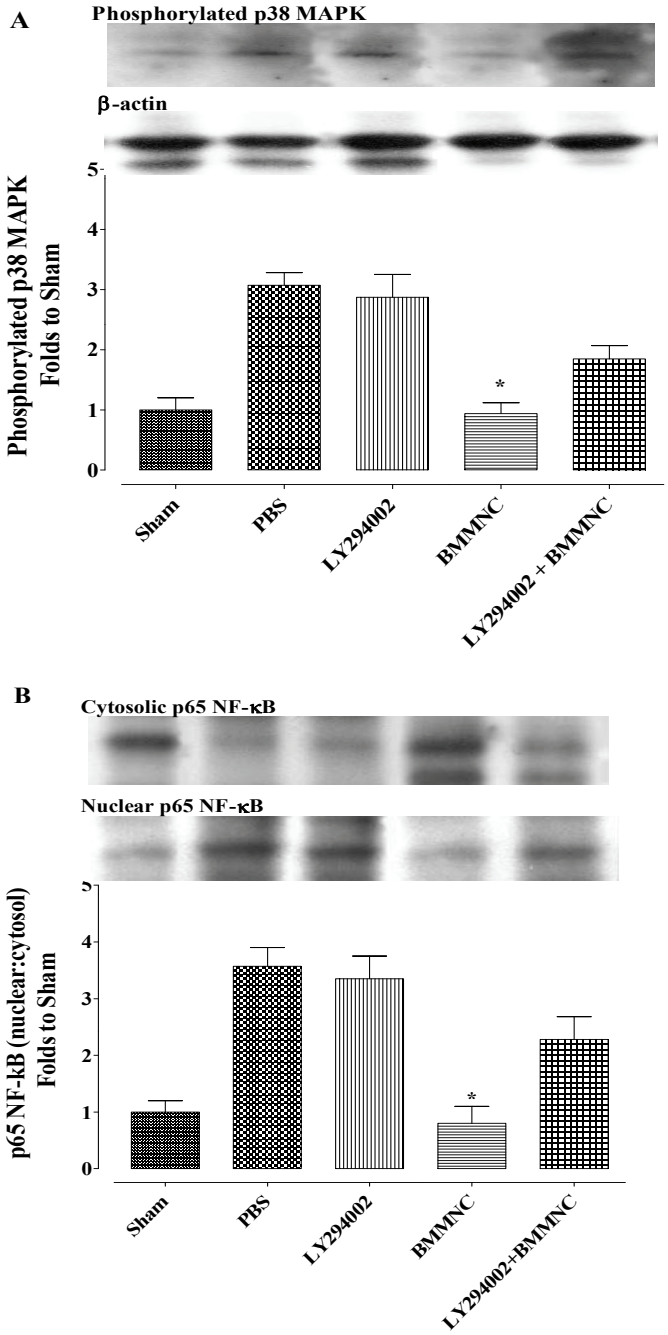


Figure 4.5.2.4 Western blots of left ventricular area at risk 30 min post LAD reperfusion to detect phosphorylated cytosolic p38-mitogen activated protein kinase (p38MAPK) and nuclear translocation of p65 nuclear factor (p65NF-κB). (**A**) When compared with phosphate buffered saline (PBS), an IV bolus of 10 million bone marrow mononuclear cells (BMMNC) upon reperfusion significantly attenuated the phosphorylation of p38MAPK. Significantly, phosphorylation of p38 MAPK was higher following pre-treatment with LY294002. (**B**) Compared to PBS, BMMNC upon reperfusion also significantly attenuated nuclear translocation of p65NF-κB, which was significantly higher following pre-treatment with LY294002 (1-way ANOVA and Dunnett's post hoc test, *P< 0.05).

4.6 Discussion

In this chapter, I have shown that in a rat model of regional I/R, IV administration of either BMMNC or BMS upon reperfusion reduced infarct size. Further, BMMNC and BMS were associated with increased expression of cardioprotective proteins. That cardioprotection was not exclusive to injection of cells suggested that BMMNC afforded cardioprotection by secreted factors. The equivalent expression of the anti-apoptotic signaling protein 14-3-3 epsilon by both BMMNC and BMS suggests the extracellular pro-survival factors activated either G-protein coupled receptors or receptor tyrosine kinases to activate the PI3K/Akt survival kinase pathway. Activated PI3K converts membrane phosphatidylinositol 4, 5 diphosphate (PIP2) to phosphatidylinositol 3,4,5 triphosphate (PIP3). Akt binds to PIP3 and is brought into close proximity with membrane bound phosphoinositide dependent kinases (PDK1 and PDK2). PDK2 phosphorylates Akt at serine-473, which then stimulates the phosphorylation of Akt at threonine-308 by PDK1 to fully activate Akt (Sarbassov *et al.,* 2005). Activated Akt phosphorylates and inactivates a myriad of pro-apoptotic molecules including Bcl-2-associated death promoter protein (BAD). Serine phosphorylation of BAD by Akt, permits 14-3-3 epsilon to bind with BAD, and prevents BAD from forming hetero-dimers with the anti-apoptotic proteins Bcl-2 and Bcl-X_L (Zha *et al.,* 1996). Free Bcl-2 and Bcl-X_L normally bind to mitochondrial voltage dependent anion channel (VDAC) to maintain the channel in a controlled open state, which allows continued

export of high energy phosphates (Vander Heiden *et al.,* 2000). Dimerization of Bcl-2 or Bcl-X_L with BAD, frees the pro-apoptotic Bax and Bak to bind with VDAC causing its disruption, mitochondrial depolarization, release of cytochrome c, and subsequent initiation of apoptosis (Shimizu *et al.,* 1999). Thus, the higher expression of 14-3-3 epsilon in the BMMNC and BMS treated hearts may have contributed to the increased expression of voltage dependent anion channel isoform 1 (VDAC-1). Moreover, VDAC-1 may have a role in the formation of mitochondrial membrane transition pore (MPTP) pore (Cesura *et al.,* 2003; Crompton *et al.,* 1998). Thus, the higher expression of VDAC-1 during reperfusion could have prevented the formation of the MPTP, subsequent mitochondrial membrane depolarization, and release of pro-apoptotic proteins (Halestrap *et al.,* 2004).

The treatment with BMMNC upon reperfusion was also associated with significant attenuation in phosphorylated p38 MAPK. Activated or phosphorylated p38 MAPK augments myocardial I/R injury (Gao *et al.,* 2002; Kaiser *et al.,* 2004) possibly by MAPK mediated phosphorylation of NHE-1 during reperfusion(Khaled *et al.,* 2001). But pre-treatment with LY294002 only partially restored phosphorylated p38 MAPK, when compared with the controls (Figure 4.5.4). This suggests that BMMNC inhibition of p38 MAPK during I/R might also be by other mechanisms such as ROS as suggested a previous report (Sabri *et al.,* 1998). Thus, BMMNC may have also attenuated the phosphorylation of p38 MAPK by

attenuating ROS possibly by BMMNC expressed anti-oxidants, which is corroborated by their higher expression as shown in the proteomic data.

The increased expression of the anti-oxidants catalase and peroxiredoxin-6, in hearts treated by BMMNC may have contributed to their cardioprotective role (Nagy *et al.,* 2006). Interestingly, in the BMS treated hearts there was only increased expression of peroxiredoxin-6. The traditional role for anti-oxidants as scavengers of ROS has, however, recently been revised as it becomes more evident that ROS play an important regulatory role in pro-survival signaling pathways. Intracellular H_2O_2 can reversibly oxidize and inhibit the lipid phosphatase PTEN (phosphatase and tensin deleted in chromosome 10), which dephosphorylates PIP3 and blocks the phosphorylation of Akt. Previous reports have suggested that some growth factors increase pro-survival signaling by increased intracellular H_2O_2 production and inhibition of PTEN (Kang *et al.,* 2004). The presented data suggests that excessive antioxidants may be unnecessary and that PI3 mediated Akt signaling is of much greater importance for attenuating I/Rinjury.

Further aspects of BMMNC mediated cardioprotection that were enlightened by the proteomic data need further discussion. HSPs have important roles as molecular chaperones by ensuring correct folding of newly synthesized proteins and repair or degradation of denatured proteins. Both BMMNC and BMS treated hearts had had increased expression of

HSP which have been suggested to protect against I/R injury: HSP20 (Fan *et al.,* 2005), alpha-B-crystallin (Morrison *et al.,* 2003), HSP72 (Suzuki *et al.,* 2002), TNF receptor associated protein-1(Montesano Gesualdi *et al.,* 2007; Xiang *et al.,* 2010), and ischemia responsive protein-94 (Yagita *et al.,* 1999). The regulation of HSP is by Heat Shock Transcription factor-1, which is subject to PI3K/Akt signaling (Zhou *et al.,* 2004).

Increased glycolytic bioenergetics is associated with reduced fatty acid oxidation and this bioenergetic switch reportedly can reduce apoptosis following myocardial ischemia and reperfusion (Opie *et al.,* 2002). This was particularly notable in BMMNC treated hearts and to some extent in BMS treated hearts. Fatty acid oxidation during reperfusion leads to lipid peroxidation and loss of mitochondrial cardiolipin, which is associated with leakage of cytochrome c from the inner mitochondrial membrane with subsequent initiation of apoptotic cell death (Paradies *et al.,* 2004). The cytoprotective metabolic switch from FAO to glycolysis by the pro-survival transcription factor, hypoxia inducible factor-1-alpha (HIF-1α). Effects of HIF-1α include the increased expression of glycolytic enzymes and suppression of fatty acid oxidation enzymes (Belanger *et al.,* 2007). During hypoxia, HIF-1α levels increase and undergo nuclear translocation, to promote transcription of pro-survival genes. Stabilization of HIF-1α during reperfusion is dependent upon activation of PI3/Akt and the concomitant increased expression of HSP72 and HSP90. Both HSP72 and HSP90 bind to the oxygen dependent degradation domain of HIF-1α and prevent its

degradation during normoxia (Zhou *et al.,* 2004). This is concordant with the data presented here.

There are a number of limitations to the data presented in this chapter. Firstly, we were not able to identify or characterise the cardioprotective composition of BMS. An attempt to separate and identify proteins in BMS by proteomics, however, produce 2-DE gel images of very low intensity spots and protein identification was not possible. Secondly, the intravenously injected BMMNC were not tracked to see whether they homed into the ischemic myocardium or became trapped in the other organs such as the lung. Thus, it is not entirely clear whether the injected BMMNC caused a local effect, a distant effect, or a combination of both.

In summary, I/R can be attenuated by IV administration of either BMMNC or BMS at the onset of reperfusion. This suggests adult stem cells have an important non-regenerative cardioprotective role which is mediated by their secreted factors. Moreover, the injection of BMMNC or BMS was associated with increased expression of cardioprotective proteins including proteins associated with PI3/Akt signaling, anti-oxidants, HSP and glycolytic proteins. The data presented here suggests that the secreted proteins of adult stem cells may be used as adjuncts to current myocardial protective strategies and could augment myocardial protection. Further, BMS may provide an "off the shelf" therapeutic option and warrants further investigation.

Chapter 5

Mesenchymal Stem Cell Therapy and Regional Myocardial I/R

5.1 Introduction

In the previous chapter, the data supported the hypothesis that non-regenerative mechanisms principally underlie cardioprotection afforded by unfractionated BMMNC therapy. Unfractionated BMMNC, however, contain three types of stem cells: haematopoietic stem cells, MSCs, and endothelial progenitor stem cells . Mesenchymal stem cells contribute only a small fraction of the total BMMNC population (< 0.01%) and are considered to be the stem cells to regenerate non-haematopoietic tissues (Prockop 1997). Mesenchymal stem cells are easily isolated from BM by their adherence to plastic. Mesenchymal stem cells are highly proliferative and can be rapidly expanded in culture, have potential for safe allogeneic cell therapy, and also have potential to differentiate into non-haematopoietic cells (Ferrari *et al.,* 1998; Friedenstein *et al.,* 1987; Makino *et al.,* 1999). These advantages suggest important benefits that have not been demonstrated in the clinical trials to date (1) allogeneic transplantation of superior quality to autologous stem cells, (2) ease of isolation and high proliferative activity may permit "off the shelf" stem cell therapy (3) cardiomyocyte differentiation to replace dead or injured cardiomyocytes. With these advantages in mind, I investigated whether bone marrow derived

MSC might be cardioprotective in a rat model of LAD myocardial ischaemia and reperfusion.

5.2 Mesenchymal Stem Cell Therapy and Regional Myocardial I/R

5.2.1 Methods

Donor MSC were isolated from BM obtained from *ex vivo* femurs and tibae of Wistar male rats. Whole bone marrow was collected in complete culture media (10ml alpha-Modified Eagles Medium (α-MEM) + 1% glutamine + penicillin 100U/ml + streptomycin 100mg/ml, and 5% foetal bovine serum) and MSC were isolated by virtue of their adherence to a plastic surface (Friedenstein *et al.,* 1987). The BM obtained from femurs and tibae was centrifuged (1500rpm for 5 min) and resuspended in culture medium for subsequent culture in T75 tissue culture flasks. Non-adherent cell were removed by repeat washes with culture media at the following times 1, 2, 6, 12, 24, 48 and 72 h. The remaining plastic adherent cells had media changes every 48 h and passage at 70% confluence by 0.25% trypsin-EDTA for 5 min in a 37^{o}C incubator with 5% CO_2. At each passage, reseeding cell density was $1.5x10^5/cm^2$. Culture expansion of plastic adherent cells was demonstrable beyond passage number 25, however, only cells obtained from cultures less than passage number 13 were used for *in vivo* experimentation. The isolated and culture expanded cells were

characterised at each passage by flow cytometric analysis for specific cell surface antigens as previously described (Chapter 2). Following culture expansion cells were mobilised: removal of the culture medium and incubation with 5ml 0.25% trypsin-EDTA for 5 min in a 37^{o}C incubator with 5% CO_2. The disassociated cells were collected in 1 ml Hank's Buffered Salt Solution (HBSS), centrifuged at 1500 rpm for 5 min, and resuspended in 0.5 ml PBS. logarithmic amplification.

Randomly selected Wistar male rats underwent 25 min LAD ischemia and 2 h reperfusion. At the onset of reperfusion, animals selectec for MSC therapy received MSC, suspended in 0.5 ml PBS, as a single IV bolus. At the end of 2 h reperfusion, the region of the left ventricle subjected to LAD ischaemia and reperfusion was analyzed for infarct size by the method previously described (Nachlas *et al.,* 1963). Vehicle controls received PBS and Sham controls underwent the same surgical manipulation, but not LAD ischaemia and reperfusion.

Table 5.2.1.1 Experimental Design: Myocardial infarct size and adult bone marrow derived mesenchymal stem cells (MSC) therapy upon reperfusion

Group	n	Reperfusion Protocol	IV Injection upon Reperfusion
Sham	6	No I/R-2h	0.5 ml PBS
PBS	8	I/R-2h	0.5 ml PBS
MSC-0.5	4	I/R-2h	0.5 $x10^6$ MSC in 0.5 ml PBS
MSC-1	5	I/R-2h	1 $x10^6$ MSC in 0.5 ml PBS
MSC-2.5	5	I/R-2h	2.5 $x10^6$ MSC in 0.5 ml PBS
MSC-5	7	I/R-2h	5 $x10^6$ MSC in 0.5 ml PBS

5.2.2 Results

In Figure 5.2.2.1 (A) The AAR (% LV) from I/R, was no different between the controls and treatment groups. Similarly, in Figure 5.2.2.2, I have shown that the haemodynamic parameters mean arterial pressure, heart rate and pressure rate index were not different between the controls and treatment groups at all time points. When compared with PBS, the injection of MSC at reperfusion did attenuate myocardial infarct size and these reductions were statistically significant particularly when the number of MSC injected were greater than one million cells (Figure 5.2.2.1 B).

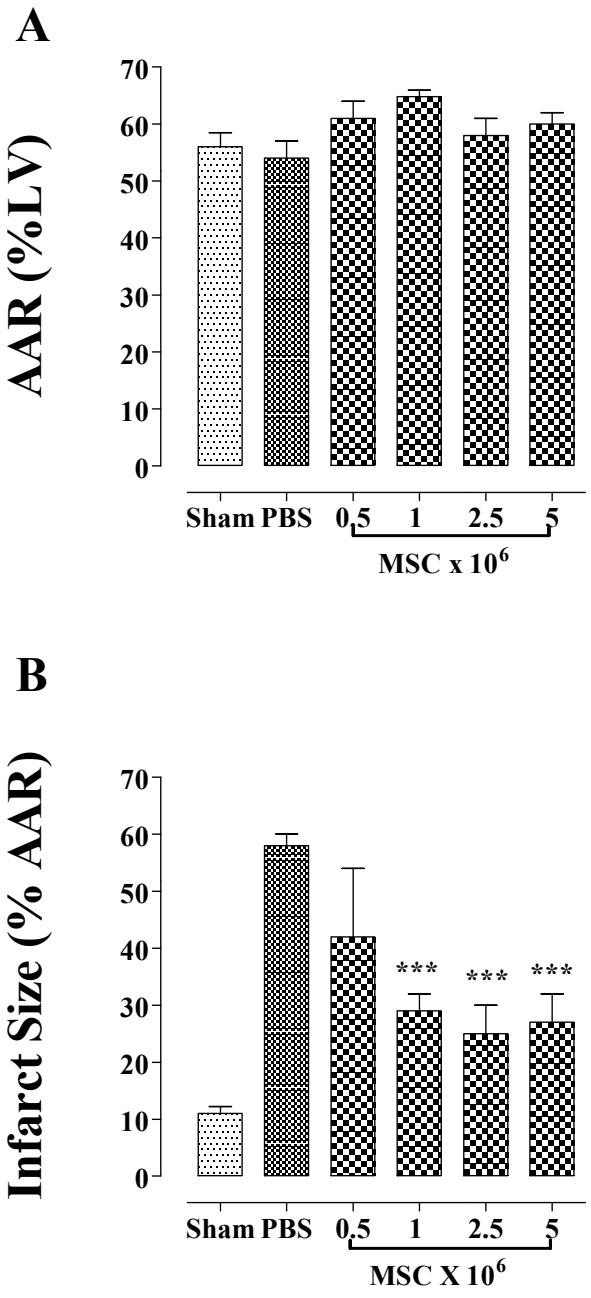


Figure 5.2.2.1 Myocardial infarct size and adult bone marrow derived mesenchymal stem cells (MSC) therapy upon reperfusion. (A) Area at risk (AAR) from 25 min LAD ischaemia and 2 h reperfusion, expressed % LV and was similar between all groups (1-way ANOVA, P>0.05). **(B)** When compared to PBS, infarct size was significantly reduced by a single IV bolus of one million or more MSC upon reperfusion (1-way ANOVA and Dunnett's post hoc test, ***P<0.0001). The sham group underwent the same surgical procedure but no LAD occlusion.

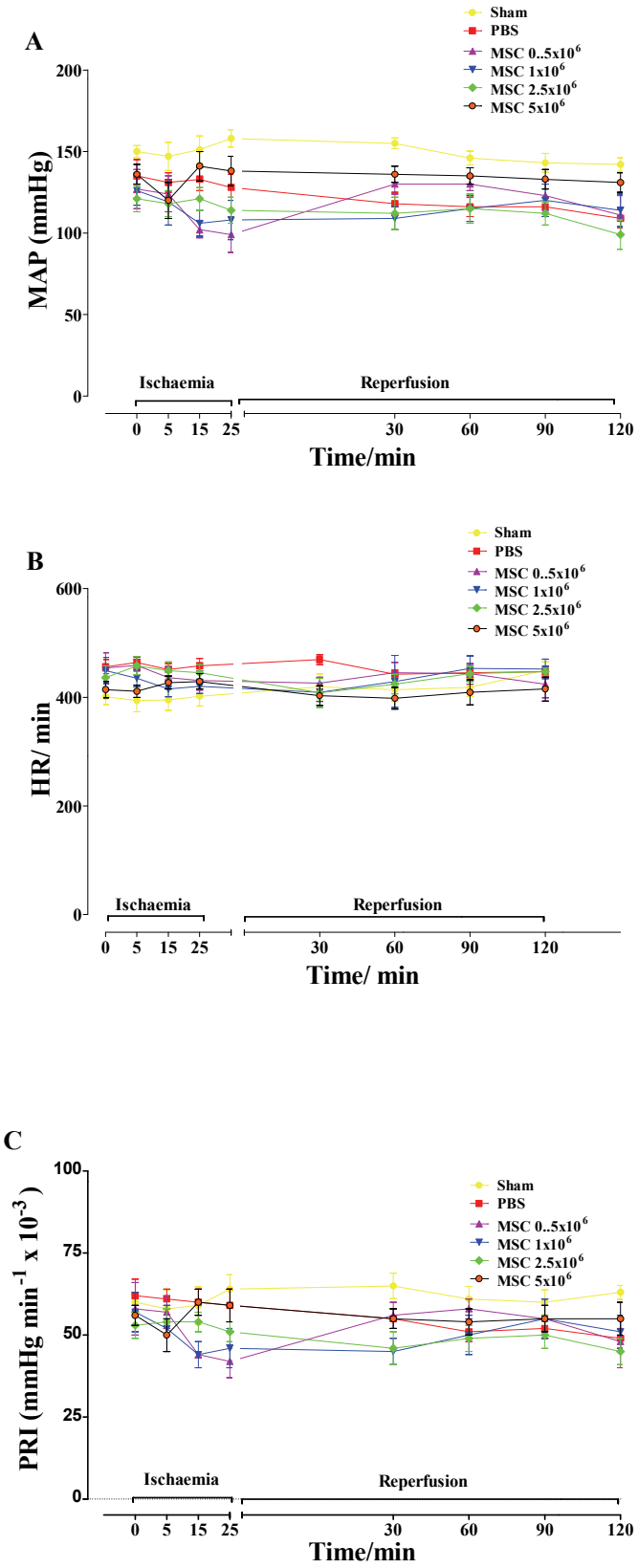


Figure 5.2.2.2 The haemodynamic parameters were similar between all experimental groups from baseline to the end of each experiment. (A) Mean arterial pressure (MAP); (B) Heart rate (HR); (C) Pressure rate index (PRI) (2-way ANOVA, $P > 0.05$).

5.3 Bone Marrow MSC Therapy and Cardiac Function post Regional I/R

5.3.1 Methods

Having shown the cardioprotective effect of IV MSC injection at the onset of myocardial reperfusion, I then investigated whether MSC injection had any effect upon cardiac function. The modalities of transthoracic echocardiography and left ventricular pressure catheter quantified indices of left ventricular function. Randomly selected Wistar male rats were subjected to 25 min of LAD occlusion and reperfusion for 7 days (I/R-7D) as described previously (Chapter 2). A delay of 7 days before the acquisition of cardiac functional data was necessary to allow for the resolution of post-ischaemic ventricular stunning (Braunwald and Kloner, 1982). I injected 2.5 million MSC as a single IV bolus at the onset of reperfusion. After thoracotomy closure and 1 hour following the onset of reperfusion the general anaesthetic was terminated and animals were recovered. At the end of 7 days post I/R-7D, echocardiography and LV pressure catheter data acquisition and analysis was undertaken by observers blinded to the experimental groups.

Table 5.3.1.1 Experimental Design: Myocardial infarct size following either IV PBS or bone marrow derived mesenchymal stem cells (MSC) in a model of 25 min LAD ischaemia and 7 days reperfusion (I/R-7D). Wistar male rats underwent either 25 min LAD occlusion and 7 days of reperfusion (I/R-7D) or no LAD occlusion i.e. sham procedure. An IV bolus of either PBS or 2.5 million MSC was at the onset of reperfusion. n is the number of animals in each group.

Group	**n**	**Reperfusion Protocol**	**IV Injection upon Reperfusion**
Sham	5	No I/R-7D	0.5 ml PBS
PBS	5	I/R-7D	0.5 ml PBS
MSC	5	I/R-7D	2.5 x10^6 MSC in 0.5 ml PBS

5.3.2 Results

In Figure 5.3.2.1, I present the data relating to left ventricular function as determined by transthoracic 2D echocardiography, following I/R-7D. When compared with controls (PBS), injection of 2.5 million MSC upon reperfusion preserved left ventricular function. Thus, both LVEF and FAC were significantly higher in the MSC therapy group.

In Figure 5.3.2.2 (A-E) data for the LV pressure transduction catheter is presented. (A-D) when compared with PBS controls, injection of 2.5 million MSC upon reperfusion did not significantly improve mean values for dP/dt_{max}, contractility index, dP/dt_{min}, and tau. (E) However, when compared with controls, LVEDP was significantly lower following MSC injection. The intraventricular pressure catheter will depend upon LV volume loading and the non-significant differences between groups for the dP/dt_{max}, contractility index, dP/dt_{min}, and tau may have been due to lower LV preloading.

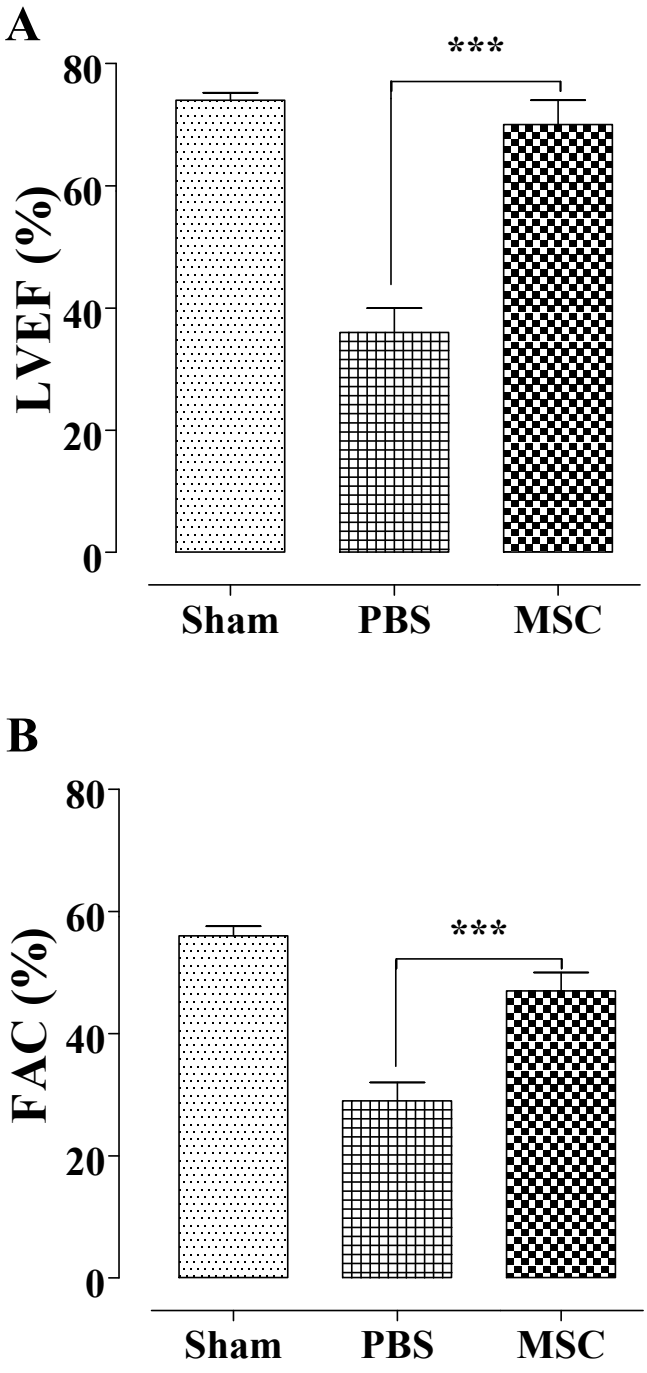


Figure 5.3.2.1 Transthoracic 2D echocardiography following I/R-7D.(A) Compared to PBS, LVEF was significantly higher in animals treated by a single IV bolus of MSC upon reperfusion (1-way ANOVA and Dunnett's post hoc test, ***P<0.0001, n=5). (B) Similarly, when compared to the PBS treated animals. Left ventricular fractional area of contraction (FAC) was significantly higher for IV injection of MSC injection (1-way ANOVA and Dunnett's post hoc test, ***P<0.0001, n=5).

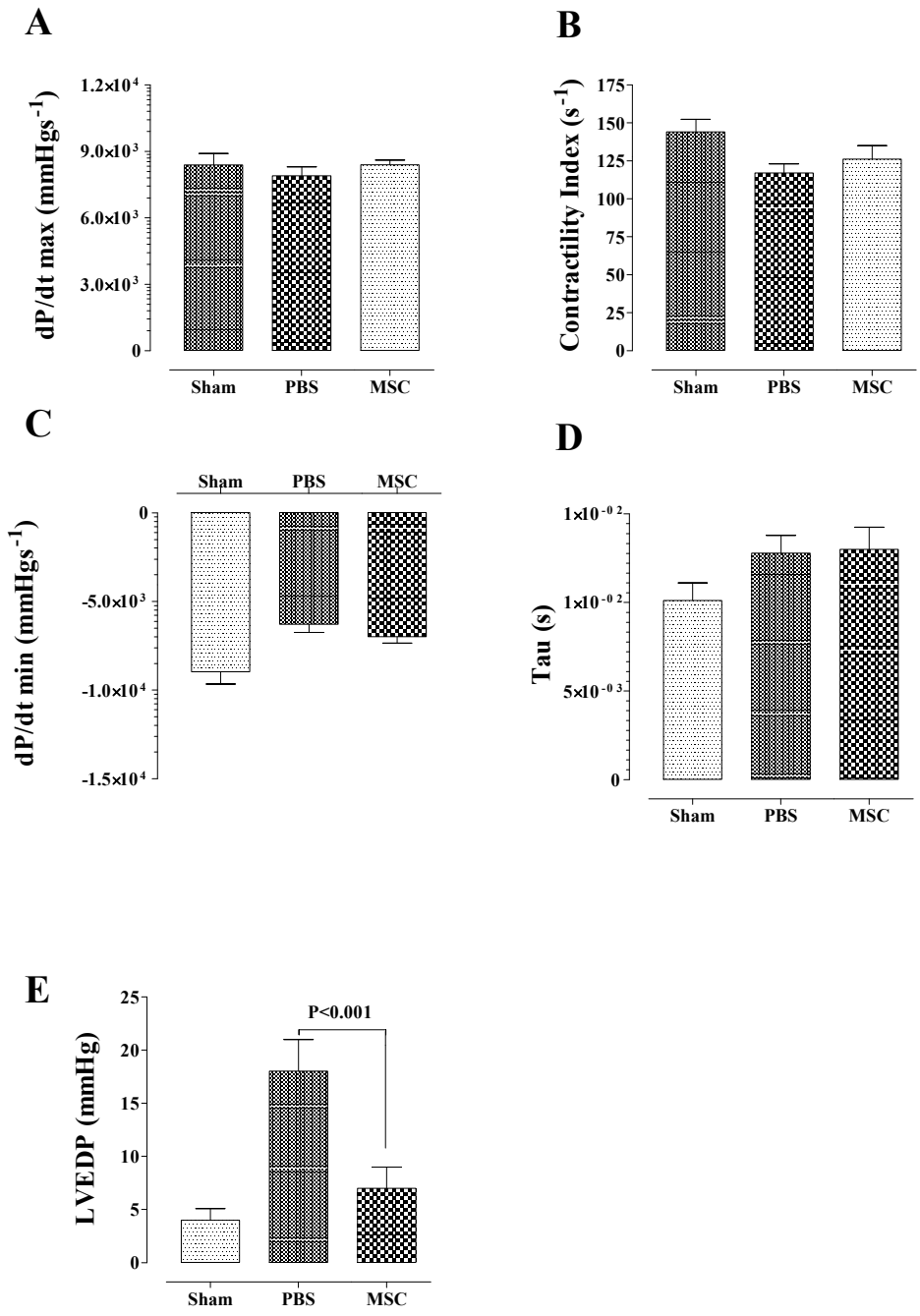


Figure 5.3.2.2 LV pressure catheterisation post I/R-7D. (**A**) When compared to PBS, dP/dt_{max} was non-significantly higher in animals treated with 2.5 million MSC, upon reperfusion. (**B**) LV contractility index was also non-significantly higher for MSC treated animals when compared to the PBS controls. **(C)** dP/dt_{min} was non-significantly lower for MSC when compared to the PBS **(D)** Tau was similar for MSC when compared to PBS. **(E)** Compared to PBS, LV end diastolic pressure (LVEDP) was significantly lower in animals treated by MSC upon reperfusion (1-way ANOVA and Dunnett's post hoc test, $P<0.001$, n=5).

5.4 Mesenchymal Stem Cell Therapy and Cardiac Fibrosis following Regional I/R

5.4.1Methods

A qualitative assessment of the volume of ECM collagen deposition post 25 min LAD occlusion and reperfusion for 21 days was by picrosirius red staining cryosections of Wistar male rat hearts. The donor 2.5 million MSC was by IV bolus at the onset of reperfusion. After 21 days of reperfusion, the rats were anaesthetized using 5% isoflurane and then sacrificed by cervical dislocation. The hearts were then excised and perfused with 10ml of ice cold PBS, followed by 5ml ice cold 4% paraformaldehyde (Sigma) via an 18 Ch cannula inserted in the ascending aorta and then immersed for 30 min in 4% paraformaldehyde on ice. Next the hearts were washed with PBS and incubated with 30% sucrose in PBS solution at 4°C overnight. The fixed hearts were then cut transversely into two segments between the base and the apex. These segments were then embedded in OCT compound (VWR) using a polyethylene mould and frozen in liquid nitrogen cooled isopentane and stored at -80°C. Cryosections (15-25μm) were cut from each segment using a rotary cryotome (Leica) and then placed on polysine-coated glass slides (VWR). Cryosections were washed with PBS and dried at room temperature overnight. The sections were then immersed in fast blue (0.15% Fast Blue in magnesium borate) for 10 min at room temperature. The sections were then washed with de-ionised water and stained with 0.1% picrosirius red F3B (0.1g Sirus red in 100ml saturated aqueous picric acid)

for 10 min at room temperature. The sections were then rinsed in de-ionised water for 5 times and then in immersed in picric alcohol (20ml, absolute alcohol; 70ml de-ionised water; 10ml saturated aqueous picric acid). Sections were then dehydrated through a methanol series and mounted in DPX (VWR) and examined by microscopy.

5.4.2 Results

Myocardial fibrosis was demonstrable by increased collagen deposition and this was identifiable by increased myocardial uptake of picrosirius red (PS). Regions with differential myocardial fibrosis are therefore remarkable under microscopic examination. When I compared the sham hearts with injection of PBS upon reperfusion, I noted LAD regional ischaemia-reperfusion injury and treatment with PBS caused an extensive degree of picrosirius red staining within the anterior myocardium of the left ventricle. Further, I noted the free wall thickness of the myocardium with increased staining with picrosirius red was much thinner, dilated LV cavity, and regions remote from increased PS staining were hypertrophied. In contrast, when I compared the picrosirius staining of the PBS treated hearts with MSC treated hearts the degree of picrosirius staining was much lower. The reduced PS staining was also associated with normal wall thickness and a non-dilated left ventricle.

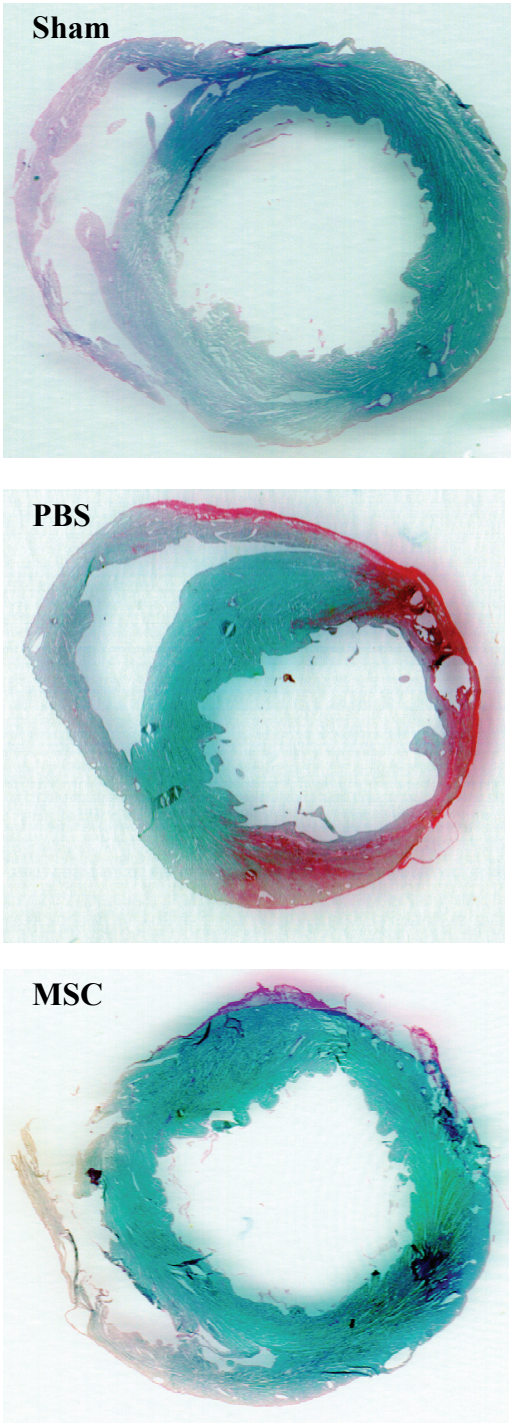


Figure 5.4.2.1 Picrosirius red staining. Cryosections of hearts from animals 21 days post 25 min LAD ischaemia and reperfusion and treated by a single IV bolus of 2.5 million culture-expanded mesenchymal stem cells (MSC) or phosphate buffered saline group (PBS) upon reperfusion. The sham heart underwent the same surgical procedure except for LAD ischaemia and reperfusion.

5.5 Discussion

In this chapter, I have shown that BM derived MSC could be culture expanded to provide non-regenerative stem cell benefits in a rat model of regional myocardial I/R. These benefits include the reduction in infarct size by 1 million or more MSC given IV upon reperfusion. In concordance with the afforded cardioprotection, there was also preservation in myocardial function as evidenced by the echocardiogram assessment of ventricular function, namely the LVEF and FAC. Although, the LV pressure catheter assessment of LV function were not entirely consistent with that noted on echo, this may be have been due to lower preloading of LV as suggested by the significantly low LVEDP. The benefits of reduction in myocardial infarct size and preservation of LV function were also concordant with the appearance of significantly reduced ECM fibrosis and absence of adverse ventricular remodeling.

There are particular advantages of using culture expanded MSC as an adult stem cell source:

1. Culture expanded bone marrow MSC might be a potential "off the shelf" source of adult stem cells, which would be desirable clinically in the emergent treatment of AMI. Clearly, the autologous harvest of BMMNC would be time consuming and harvested BMMNC might not be available for administration at the onset of coronary reperfusion, in a case of AMI.

2. That allogeneic MSC injection might be feasible has been suggested by data that suggests that MSC are "immune-privileged" and are unlikely to cause a host immune reaction (Meisel *et al.,* 2004). Further, clinical data from bone marrow transplant patients suggest allogeneic and donor mismatch MSC injections are beneficial in steroid refractory graft versus host disease (Le Blanc *et al.,* 2008).
3. Autologous bone marrow MSC might be harvestable and culture expanded well in advance of a cardiac procedure where a significant period of regional or global myocardial reperfusion injury is anticipated, e.g. complex open-heart surgery.
4. Autologous bone marrow MSC harvested well in advance might be optimised for improved non-regenerative and possibly regenerative benefits, e.g. MSC over expressing Akt (Gnecchi *et al.,* 2006).

Chapter 6

Trafficking of Stem Cells during Regional Myocardial I/R

6.1 Introduction

Thus far, I have shown that a single IV bolus of exogenous BM derived stem cells when injected at the onset of reperfusion can attenuate myocardial reperfusion injury. There is, however, a vast endogenous stem cell reservoir contained within the bone marrow architecture of the entire endoskeleton. The mobilisation of bone marrow stem cells is by signaling between stromal cell derived factor-1 (SDF-1) and its cognate receptor CXCR4. Endothelial and stromal cellular lining of the bone marrow stem cell niche constitutively expresses SDF-1, which interacts with its cognate receptor CXCR4 expressed on the stem cell surface (Dar *et al.,* 2005; Imai *et al.,* 1999; Ponomaryov *et al.,* 2000). Further, signaling between bone marrow vascular niches expressing SDF-1 and haematopoietic stem cell surface expressed CXCR4 signaling contributes to the retention and quiescence of haematopoietic stem cells in their endosteal niches (Nie *et al.,* 2008). During homeostasis, bone marrow stem cells continually and variably egress into the blood stream in a circadian cyclical manner. These circadian changes in the levels of circulating bone marrow stem cells is under sympathetic control via noradrenaline binding to beta-3-adrenoceptors on bone marrow stromal cells to decrease expression of SDF-1 and reduced

CXCR4 signaling (Mendez-Ferrer *et al.,* 2008). These daily changes in bone marrow stem cell mobilisation are, however, slow and peak 5 h following exposure to light (Mendez-Ferrer *et al.,* 2008). In stress or pathological states, e.g. following AMI there is an elevation of SDF-1 in the heart, which intravasates to create a chemokine or SDF-1 gradient between the bone marrow stem cell niche and site of the myocardial infarction for trafficking of mobilised stem cells (Askari *et al.,* 2003; Ceradini *et al.,* 2004; Jiang *et al.,* 2002; Wojakowski *et al.,* 2004). Taking into account that SDF-1 is highly expressed by the bone marrow stem cell niche (Sugiyama *et al.,* 2006), a rapid and massive intravasation of bone marrow stem cells would require a commensurate reduction in SDF-1 activity within the bone marrow niche. The data that I have presented thus far suggests that the endogenous bone marrow stem cell mobilisation cannot match the timing and magnitude of an exogenous bone marrow stem cell delivery. Direct disruption of CXCR4 and SDF-1 interaction, however, can lead to a massive and rapid mobilisation of endogenous bone marrow stem cells (Broxmeyer *et al.,* 2005; Sweeney *et al.,* 2002). An important endogenous mechanism that leads to the direct disruption of CXCR4 and SDF-1 interaction and de-adhesion of stem cells from their niche is the stem cell expression of CD26. CD26 is a type II transmembrane glycoprotein with extra-membranous dipeptidyl peptidase-4 activity to cleave and inactivate SDF-1 (Zhai *et al.,* 2009). Moreover, the reduced expression of CD45 expression leads to impairment of SDF-1 function, which can also contribute to a reduction in

stem cell retention within the bone marrow (Shivtiel *et al.,* 2008). In this chapter, I have investigated modulations in the endogenous bone marrow mononuclear cell phenotype in the context of regional myocardial ischaemia-reperfusion injury. In chapter 3, I presented data that demonstrated equivalent protection by exogenous BMMNC injection and ischaemic preconditioning. Thus, I examined whether myocardial ischaemic preconditioning modulated the endogenous bone marrow stem cell phenotype, given that it is the most powerful endogenous myocardial protective strategy against I/Rinjury, to date (Murry *et al.,* 1986).

6.2 Modulation of Endogenous BMMNC Phenotype post Regional Myocardial I/R

6.2.1Methods

Randomly selected Wistar male rats were subjected to I/R-2h and subsequently underwent BM removal from femurs and tibae, as previously described (Chapter 2). Sham controls underwent the same surgical procedure but no LAD occlusion. BMMNC were isolated from the BM by Percoll density gradient centrifugation, as previously described. Primary monoclonal antibodies for cell surface markers CD34, CD45, CD133, c-Kit, were separately added to one million BMMMC aliquots for 30 min on ice. BMMNC were washed twice with 1 ml HBSS, centrifugation (1500 rpm for 5 min), resuspended in 100 µl HBSS and the secondary antibody incubation was added and cell kept for 30 min on ice and washing twice with HBSS (1 ml). BMMNC were fixed in 2% paraformaldehyde PBS (500 µl) and

analyzed by fluorescence activated cell sorting (FACS) flow cytometry using a DakoCyan flowcytometer equipped with Summit v4.3 software (DakoCytomation, UK). FACS data collected 10,000-gated events using 3-decade logarithmic amplification.

6.2.2 Results

When compared with sham animals, there was a non-significant elevation in the endogenous expression of CD34, CD133 and c-Kit positive bone marrow mononuclear cell in animals subjected to 25 min LAD ischaemia and 2 h reperfusion (I/R). This modulation in endogenous phenotype effect is lower when animals subjected to I/R and receive a single IV injection bolus of exogenous 10 million *ex vivo* unfractionated BMMNC. Interestingly, *w*hen compared with the shams, animals subjected to I/R had a significantly higher expression of the endogenous bone marrow CD45 positive cells, which was lower in those animals subjected to I/R and receiving BMMNC upon of reperfusion. This lowered expression of endogenous bone marrow CD45 positive cells by the injection exogenous BMMNC upon of reperfusion, however, did not achieve statistical significance (Figure 6.2.2.1). Thus in summary, I/R increased the endogenous bone marrow expression of CD34, CD133, c-Kit and CD45 positive mononuclear cells, where only the augmented expression of CD45 positive cells was statistically significant. Secondly, the higher endogenous bone marrow expression of CD34, CD133, c-Kit and CD45 positive mononuclear cells caused by I/R was reducible by the administration of

exogenous BMMNC, but these reductions were not statistically significant. These results suggest that endogenous bone marrow stem cell phenotype changes in response to myocardial I/R injury and that a timely injection of exogenous BMMNC may be significantly beneficial to the endogenous bone marrow response to myocardial injury.

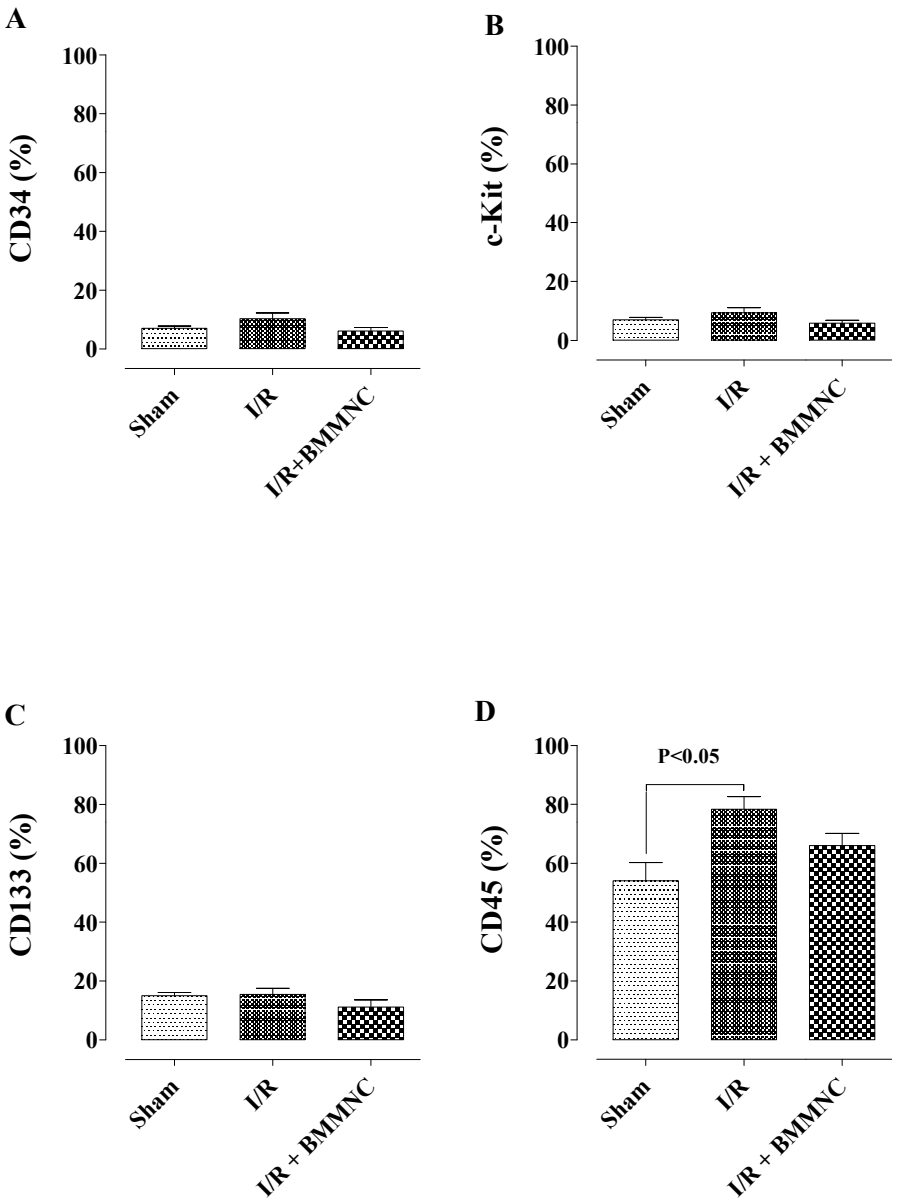


Figure 6.2.2.1 Flow cytometry phenotype analyses for the haematopoietic stem cell markers CD34, CD133, CD45 and c-kit of *ex vivo* endogenous BMMNC. (A, B&C) When compared with sham, 25min LAD ischaemia and 2 h reperfusion (I/R) non-significantly elevated CD34, c-kit and CD133 positive endogenous BMMNC. The increase in endogenous CD34, CD133 and c-kit positive cells following I/R was non-significantly attenuated by a single IV bolus of 10 million *ex vivo* unfractionated BMMNC. **(D)** When compared with shams, I/R-2h significantly augmented endogenous BMMNC CD45 positive phenotype (1-way ANOVA and Dunnett's post hoc test, $P< 0.05$, n=10).

6.3 Modulation of Endogenous BMMNC Phenotype post Ischaemic Preconditioning and Regional Myocardial I/R

6.3.1 Methods

Randomly selected Wistar male rats underwent 2 cycles of 5 min LAD ischemia and 5 min of reperfusion for ischaemic preconditioning (IPC) of the myocardium. Subsequent to the IPC, animals underwent I/R-2h. Sham controls underwent the same surgical procedure but no LAD occlusion. At the end of reperfusion, BMMNC were isolated from the BM of femurs and tibae, as previously described (Chapter 2). Primary monoclonal antibodies for the cell surface markers CD34, CD45, CD133, c-Kit, CD26, and CXCR4 were separately added to one million BMMMC aliquots for 30 min on ice. BMMNC are washed twice with 1ml HBSS, centrifugation (1500 rpm for 5 min), resuspended in 100 µl HBSS and the secondary antibody incubation was added and cell kept for 30 min on ice and washing twice with HBSS (1ml). BMMNC were fixed in 2% paraformaldehyde PBS (500 µl) and analyzed by FACS flow cytometry using a DakoCyan flowcytometer equipped with Summit v4.3 software (DakoCytomation, UK). FACS data collected 10,000-gated events using 3-decade logarithmic amplification.

6.3.2 Results

When compared with sham, animals subjected to IPC followed by I/R-2h had a non-significant elevation in the endogenous expression of CD34 and c-Kit positive BMMNC. Endogenous BMMNC expression of CD133 was similar between groups. Interestingly, when compared with sham group, endogenous BMMNC expression of CD45 was significantly lower in those animals subjected to IPC followed by I/R-2h (Figure 6.3.2.1). Further, when compared to sham animals, following IPC and I/R-2h caused endogenous BMMNC to highly express both CD26 and CXCR4; CD26 expression was at least six folds higher whilst CXCR4 expression was 10 folds higher (Figure 6.3.2.2)

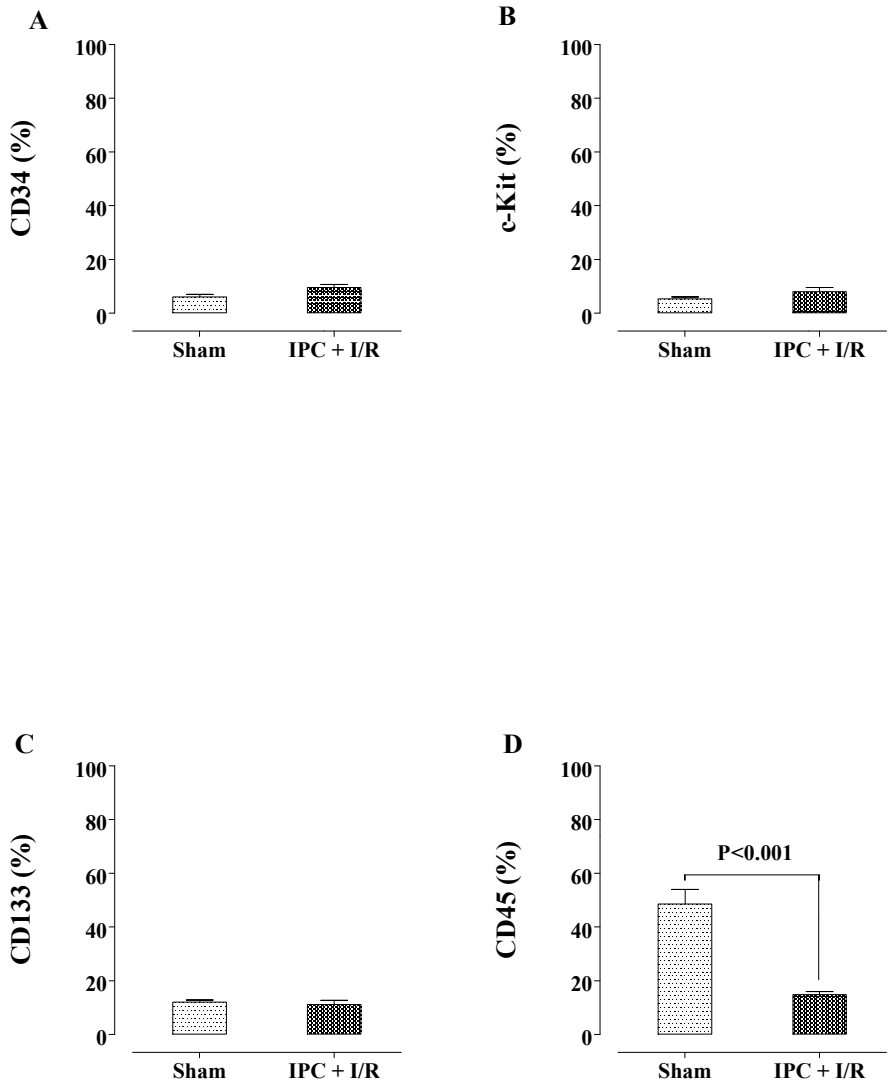


Figure 6.3.2.1 FACS flow cytometry phenotype analyses for the haematopoietic stem cell markers CD34, CD133, CD45 and c-Kit in endogenous BMMNC. (A & B) When compared with sham, ischaemic preconditioning followed by 25min LAD ischaemia and 2 h reperfusion (IPC+I/R) non-significantly elevated CD34 and c-kit expression. **(C)** Compared to sham, IPC+I/R had similar expression of CD133 positive endogenous bone marrow mononuclear cells. **(D)** When compared with shams, IPC+I/R significantly attenuated the endogenous CD45 positive endogenous bone marrow mononuclear cells (unpaired Student's t test, $P < 0.001$, n=5).

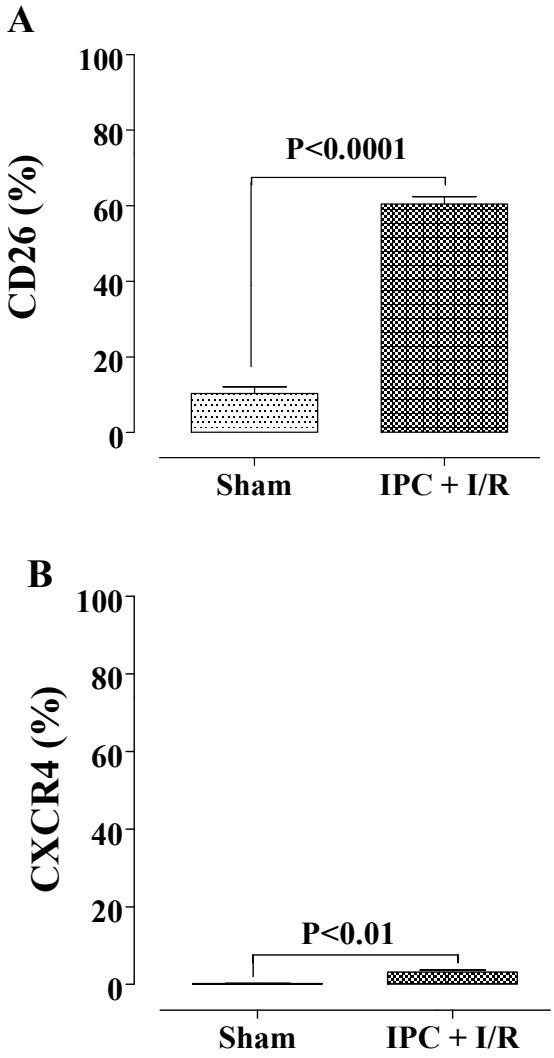


Figure 6.3.2.2 Flow cytometry phenotype analyses for the expression of endogenous stem cell trafficking surface markers CD26 CXCR4 in endogenous BMMNC. (A) When compared with sham, ischaemic preconditioning (IPC) followed by 25min LAD ischaemia and 2 h reperfusion (I/R) significantly augmented CD26 expression in the endogenous BMMNC (unpaired Student's t test, $P < 0.0001$, n=5). **(B)** In concordance, compared to sham, IPC+I/R significantly augmented expression of CXCR4 expression in the endogenous BMMNC (unpaired Student's t test, $P < 0.01$, n=5).

6.4 Discussion

In this chapter, I have presented data that demonstrates that the endogenous bone marrow derived BMMNC are capable of modulating their phenotype in response to the stress of regional myocardial ischaemia and reperfusion injury. This data is in concordance with previous data in that it suggests that the bone marrow is a dynamic system with an important role for the provision of stem cells to the repair of tissue injury (Kollet *et al.,* 2003). Firstly, I have shown that regional myocardial ischaemia and reperfusion caused the bone marrow derived BMMNC expression of CD45 to increase, which would be in accordance to the rise of inflammatory cytokines in the circulation (Lapidot *et al.,* 2002). This augmented BMMNC CD45 expression suggests an an inflammatory response by the bone marrow following I/R. Thus, the response to regional myocardial ischaemia and reperfusion injury is to increase mobilisation of haematopoietic stem cells and production of leucocytes. Secondly, I have shown that the injection of 10 million exogenous bone marrow derived BMMNC caused the endogenous bone marrow derived BMMNC to decrease expression of CD45. This non-significant but definite trend suggests that the injection of exogenous BMMNC acted to reduce the degree of myocardial injury to ischaemia and reperfusion and consequently the cross talk between the circulating blood and the bone marrow may have been to attenuate the inflammatory cellular output from the bone marrow. I do not present data to show that the attenuated CD45 positive endogenous bone marrow cells

translated to a decreased leukocyte efflux and trafficking to the ischaemic regions of the myocardium. Nevertheless, it would be reasonable to assume that a significantly reduced inflammatory cellular influx into the ischaemic and reperfused myocardium may have contributed, at least in part, to the observed attenuation of myocardial injury.

Myocardial ischaemic preconditioning followed by 25 min LAD ischaemia and 2 h reperfusion altered the endogenous bone marrow mononuclear cellular composite phenotype. Interestingly, similar to the bone marrow cellular modulation caused by an exogenous BMMNC injection, ischaemic preconditioning also caused a reduction in endogenous bone marrow expression of CD45 positive mononuclear cells. Ischaemic preconditioning, however, caused an approximate three folds reduction in CD45 expression, which was statistically significant. This suggests that the crosstalk between preconditioned myocardium and the endogenous bone marrow would be to reduce the bone marrow production and subsequent discharge of inflammatory cells, whilst augmenting C34 and c-kit positive cells. In concordance with the augmented C34 and c-kit positive endogenous bone marrow mononuclear cell expression, the communication between the preconditioned myocardium also resulted in increased bone expression of CD26 that was least six folds greater than that for the sham animals. The increase in CD26 expression was also associated with a higher expression of CXCR4 that was approximately 10 fold higher than for the bone marrow mononuclear cells from sham animals. Stem cell expression of

CXCR4 can be rapidly increased by externalization of intracellular stores on human CD34 positive cells leading to increased SDF-1 mediated trafficking (Kollet *et al.,* 2003; Peled *et al.,* 1999). These findings are in concordance with the clinical use of granulocyte colony stimulating factor (G-CSF) for mobilization of haematopoietic stem cells by decreasing SDF-1 expression and up regulating CXCR4 in human and murine bone marrow (Petit *et al.,* 2002). Stem cell mobilisation by G-CSF, however, is slow and takes approximately 5 days to peak. The magnitude of increased CD26 expression would suggest a more direct inhibition of SDF-1 within the bone marrow stem cell niche. Direct SDF-1 and CXCR4 disruption would lead to a rapid and massive increase in the circulating bone marrow derived stem cells as previously documented (Broxmeyer *et al.,* 2005; Sweeney *et al.,* 2002). A direct disruption of SDF-1 and CXR4 bonding as suggested by the six-fold CD26 expression would be consistent with a massive mobilization of endogenous bone marrow stem cells, which could be on par with an exogenous bolus injection of BMMNC. This may also explain the parity of cardioprotection when comparing ischaemic preconditioning and exogenous bone marrow stem cell injections at the onset of reperfusion, as suggested by the data I presented in chapter 3.

Taken together, these findings are concordant with the data that I have presented and the data from others reporting the phenomena of cardioprotection associated with ischaemic preconditioning, which was initially reported by Murry *et. al. (Murry et al., 1986)*. Thus, the data

presented here suggests that the cardioprotection afforded by ischaemic preconditioning may have role for highly augmented mobilization of endogenous bone marrow derived CD34 and c-kit positive stem cells, which may be essential for limiting and reparation of myocardial injury due to ischaemia and reperfusion. Moreover, a mechanism of cardioprotection by the rapid and massive mobilization of bone marrow derived stem cells might also explain the comparable myocardial protection afforded by myocardial ischaemic preconditioning, remote ischaemic preconditioning and post-conditioning.

Chapter 7

Mesenchymal Stem Cell Therapy and IPC are Synergistic

7.1 Introduction

In chapter 3, I presented data that suggested that cardioprotection by an exogenous bone marrow derived stem cell injection is comparable to myocardial ischaemic preconditioning. In the previous chapter, I have presented further data that suggests that myocardial ischaemic preconditioning may have a role in the swift and massive mobilization of endogenous bone marrow derived haematopoietic stem cells. The exact mechanism for cardioprotection afforded by ischaemic preconditioning is not entirely clear in the literature, despite its discovery more than 25 years ago (Murry *et al.,* 1986). A recent review by Yellon et. al., had suggested that the molecular mechanism underlying ischaemic preconditioning might be due to the activation of survival kinase signaling (Yellon *et al.,* 2007). In conjunction with the data presented in chapter 4, which suggests that the exogenous injection of bone marrow derived stem cells activate PI3K/Akt survival kinase pathway. I hypothesized that the underlying mechanisms for cardioprotection afforded by both exogenous bone marrow stem cell therapy

and ischaemic preconditioning are reliant upon a massive deployment of bone marrow derived stem cells within the systemic circulation. I tested this hypothesis by investigating whether the cardioprotection afforded by ischaemic preconditioning might augment the cardioprotection afforded by exogenous bone marrow derived MSC injection at the onset of reperfusion, in an anaesthetised Wistar male rat model of regional myocardial ischaemia and reperfusion.

7.2 IPC plus Mesenchymal Stem Cell Therapy and Infarct Size post Regional Myocardial I/R

7.2.1Methods

Randomly selected Wistar male rats underwent 25 min LAD ischaemia and 2 h reperfusion. Donor MSCs were isolated from BM obtained from *ex vivo* femurs and tibae of Wistar male rats, as described previously (Chapter 2). The isolated and culture expanded cells were phenotyped by FACS flow cytometry for CD34, CD133, CD45, c-Kit, CD29, and CD90 as previously described (Chapter 2). Following culture expansion, cells were mobilised by removal of culture media and deadhesion with 5 ml 0.25% trypsin-EDTA for 5 min in a 37^{o}C incubator with 5% CO_2. The disassociated cells are collected in 1ml Hank's Buffered Salt Solution, centrifuged 1500 rpm for 5 min, and resuspended in 0.5 ml PBS. Randomly selected syngeneic Wistar male rats were subjected to 25 min LAD occlusion and 2 h of reperfusion (I/R-2h). At the onset of

reperfusion, MSC therapy animals received 2.5 million MSC by IV bolus injection. At the end of 2 h reperfusion, the region of the left ventricle subjected to I/R-2h was analyzed for myocardial infarct size, as previously described. The controls received PBS and Sham animals underwent the same surgical manipulation as the other animals except for LAD occlusion and reperfusion.

7.2.2 Results

With reference to figure 7.2.2.1, the comparison of the sham animal group with the animals receiving PBS at reperfusion demonstrated a significantly increased left ventricular infarct size (Sham: 7± 0.9% versus PBS: 55 ± 2%, $P< 0.0001$, $n = 9$). Conversely, when comparing the PBS group with the animals that were subjected to IPC, there was a significant reduction in left ventricular infarct size (PBS: 55± 2%, versus IPC:30 ± 3% $P< 0.0001$, $n = 6$). The injection of 2.5 million exogenous MSCs at the onset of reperfusion consistently reduced left ventricular infarct size as compared to the PBS controls (PBS: 55± 2%, versus MSC: 25 ± 5% $P< 0.0001$, $n = 5$). Interestingly, when compared with the PBS controls, animals that had received 2.5 million cells pre-ischaemia and a further 2.5 million MSC at the onset of reperfusion (MSC+MSC) also had a significant reduction in left ventricular infarction, but this was similar to that in animals receiving MSC only upon reperfsuion. Thus, there was, however, no additional benefit conferred by the extra injection of 2.5 million MSC prior to the onset of

ischaemia (PBS: 55± 2%, versus MSC+MSC: 23 ± 10% P< 0.0001, n = 5). As hypothesized, when I compared PBS controls with animals that underwent IPC and then received 2.5 million exogenous MSC at the onset of reperfusion (IPC+MSC), there was a dramatic reduction in left ventricular myocardial infarction, similar to that in the sham animal group (PBS: 55± 2%, versus IPC+MSC: 6 ± 2% P< 0.0001, n = 7). There were no significant differences in the left ventricular area at risk between the controls and the treatment groups. There were also no demonstrable difference in the haemodynamic parameters between the controls and treatment groups (Figure 7.2.2.2).

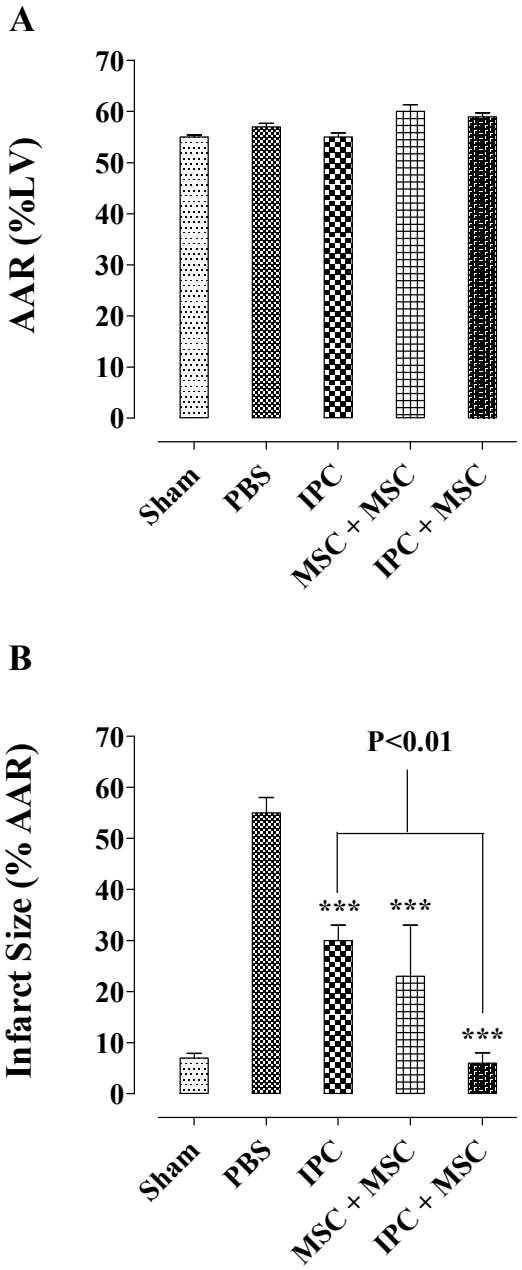


Figure 7.2.2.1 (A) The area at risk (AAR) from 25 min LAD ischaemia and 2 h LAD reperfusion, expressed as % LV was similar between all groups (1-way ANOVA, P>0.05). **(B)** When compared with controls (PBS), IPC, pre-treatment with an IV bolus of 2.5 million MSC and then a further IV bolus of 2.5 million MSC upon reperfusion (MSC+MSC), and IPC+MSC upon reperfusion significantly attenuated infarct size (1-way ANOVA and Dunnett's post hoc test, ***P<0.001, n=5). When comparing IPC with IPC+MSC, there was a further significant reduction in infarct size beyond that by IPC alone (P<0.01, n=7). The sham group underwent the same surgical procedure except for LAD ischaemia and reperfusion.

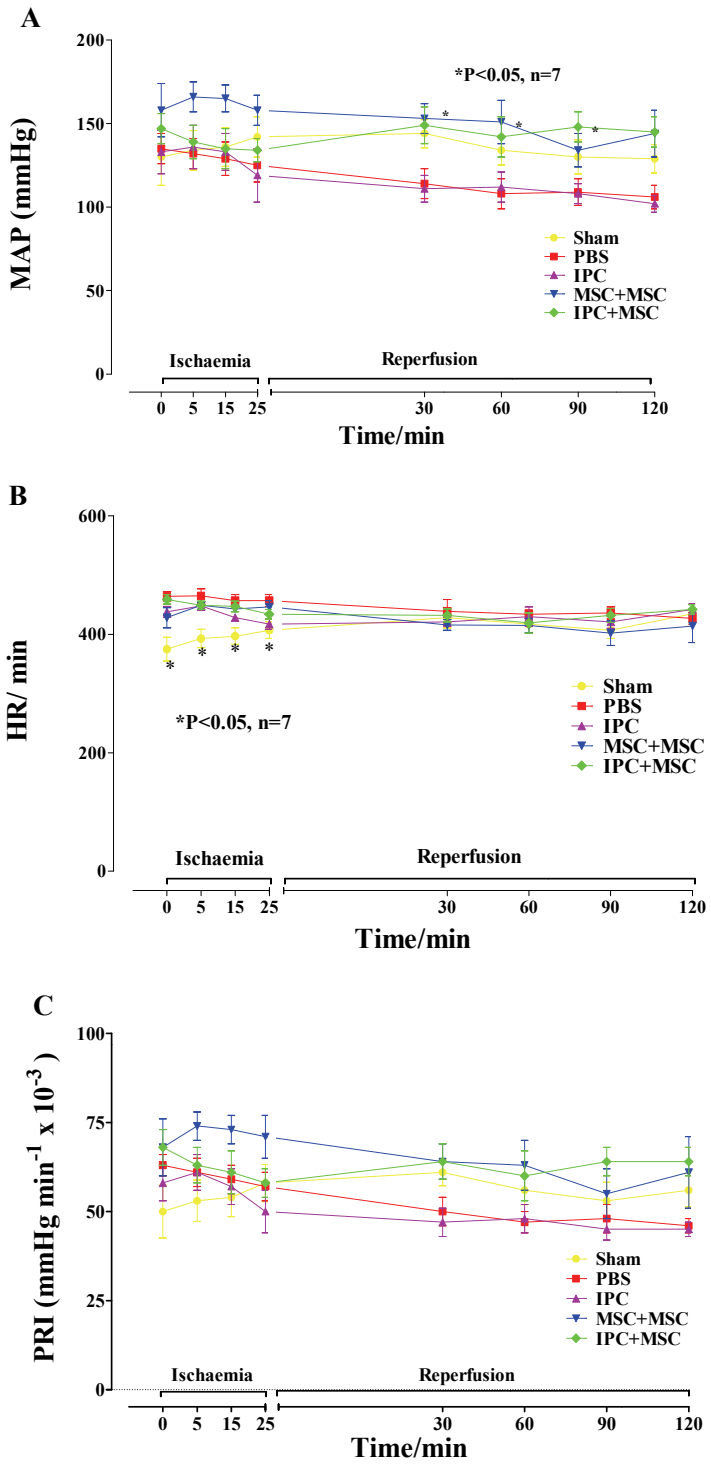


Figure 7.2.2.2 (**A**) MAP was significantly higher at reperfusion times: 60min for MSC+MSC, 90min and 120min for IPC+MSC group. (**B**) Compared to PBS, HR was significantly lower in shams at ischaemia times 0, 5, 15 and 25min. (**C**) PRI were similar between all experimental groups from baseline to the end of each experiment (2-way ANOVA and Bonferonni's post hoc test, $P > 0.05$). Sham group under the same surgical procedure except for I/R.

7.3 Discussion

In this chapter, I have presented data that tested the hypothesis that cardioprotection afforded by either ischaemic preconditioning or exogenous bone marrow derived stem cell therapy is reliant upon a swift and massive mobilization of stem cells into the peripheral circulation. I have presented data where the combination of IPC with the systemic injection of exogenous bone marrow derived MSCs upon reperfusion reduced myocardial infarct size to sham levels. The magnitude of cardioprotection afforded suggests that the two cardioprotective strategies were acting in synergy. These data also suggest the injection of 2.5 million exogenous MSC prior to the onset of reperfusion did not have any additional benefit to that afforded by the timely injection at the onset of reperfusion. The reason why this may be so is that a lack of SDF-1 expression by the myocardium prior to the onset of ischaemia may have lead to the exogenous MSC trafficking to tissues with the higher constitutive expression of SDF-1 such as the bone marrow or the spleen. It thus follows, that the haematopoietic stem cells mobilized by ischaemic preconditioning and the injected MSC may have trafficked to a high SDF-1 gradient created by the ischaemic myocardium.

IPC is the phenomenon attributable to the diligent study by Murry, Jennings and Reimer in 1986 and since its initial description it has become one of the most quoted scientific studies in the cardiovascular literature (Murry *et al.,* 1986). The following data from this landmark study corroborate the data that I have presented in this and the previous chapter.

(1) Murry *et. al.,* reported that preconditioning the heart was only cardioprotective in the short or 40 min ischaemia model but not in the 3 h ischaemia model. When taken together with my data, it suggests that the bone marrow derived stem cells were mobilised into the circulation by ischaemic preconditioning. However, in the absence of reperfusion within the first 2 h or so and in the absence of an SDF-1 chemokine gradient, the mobilized stem cells would have trafficked back to the bone marrow. Hence, the lack of protection observed in the 3 h ischaemia model. (2) The pattern of injury described in the 40-minute ischaemia model is patchy and diffuse, which is consistent with the pattern of exogenous stem cell engraftment into the myocardium following IV injection, which is consistent with observations in my laboratory and that by others. In contrast, the pattern of injury in the 40 min controls and 3h ischaemia model had solid or confluent infarcts. (3) Murry *et. al.,* also report that pre-treatment with verapamil in the 40 min ischaemia and reperfusion model was significantly cardioprotective, but continuous verapamil initiated 15 min after the onset of ischaemia in the 3 h ischaemia model was not cardioprotective. Following this observation, the authors suggest that verapamil pre-treatment might share a common mechanism with IPC. Interestingly, a more recent report suggests that verapamil can disrupt the SDF-1/CXCR4 signaling axis in bone marrow derived stem cells and impair *in vitro* MSC migration (Wang *et al.,* 2008). Thus, pre-treatment with verapamil may lead to stem cell mobilization from the bone marrow;

however, continuous verapamil injection could impair stem cell trafficking to the ischaemic myocardium. Indeed, blocking the CXCR4 receptor disrupts the SDF-1/CXCR4 cell-cell adhesion but the mobilized cells cannot home to tissues highly expressing SDF-1, such as lung, liver and bone marrow (Burger *et al.,* 2009; Liang *et al.,* 2004).

Following the description of myocardial IPC, there have been consistent reports of preconditioning the heart in numerous *ex vivo* and *in vivo* experimental models and clinical scenarios. In fact, preconditioning the myocardium can be by pharmacological and non-pharmacological stimuli that not necessarily require an initial ischaemic myocardial event. More recently, the phenomenon of remote preconditioning where the ischaemic event is distant to the target organ has been reported.

Chapter 8

8.1 General Discussion

Adult stem cell therapy to regenerate myocardial infarction thus far remains a clinical non-entity despite the passage of more than a decade since the promise of the earliest experimental reports. Whether this potential benefit of adult stem cell therapy becomes a reality of future clinical practice remains uncertain. In the course of this thesis, I have investigated another potential use of adult stem cell therapy namely the non-regenerative benefits of adult bone marrow derived stem cell therapy in a rat model of acute myocardial ischaemia and reperfusion. The initial work demonstrated that unfractionated BMMNC when applied as early as the onset of reperfusion can powerfully attenuate acute myocardial infarction, preserve myocardial function, and prevent adverse ventricular remodeling. This degree of myocardial protection was similar to that afforded by IPC, which is the undisputed gold standard for myocardial protection.

The initial experiments investigated whether infarct size could be attenuated by adult bone marrow derived stem cells or BMMNC therapy. The unfractionated BMMNC were phenotypically positive for the surface markers CD34, CD133, c-Kit, which are typically expressed by bone marrow haematopoietic stem cells. Donor BMMNC cell counts were high for CD45 phenotype, which is a pan leukocyte marker used to identify T or

B-lymphocytes within the haematopoietic progenitors. This might appear to be counter-intuitive for myocardial protection, considering that the injected CD45 positive cell population could differentiate into inflammatory macrophages and exacerbate I/R injury. In contrast, BMMNC injection afforded myocardial protection, this suggesting that lineage differentiation of donor BMMNC into an inflammatory cells was unlikely during acute reperfusion. Although at this stage I could not entirely dismiss BMMNC transdifferentiation into *de novo* myocardium there were, however, a number of aspects that did not support BMMNC differentiation to be the principal mechanism responsible for myocardial protection. Firstly, the injected BMMNC had not been enriched for c-Kit postive BMMNC, which was considered to be necessary in the initial myocardial regeneration studies using adult bone marrow derived BMMNC as reported by Orlic *et. al.,* and Jackson *et. al.,*(Jackson *et al.,* 2001; Orlic *et al.,* 2001). Secondly, the length of time following cell injection and the observed benefit at the 2 h endpoint could not support BMMNC differentiation into functional cardiomyocytes as the primary mechanism for infarct size reduction. Orlic *et. al.,* reported regeneration of myocardium 9 days post cell injection and currently there is no data to suggest that adult stem cells can differentiate into fully functional cardiomyocytes within 2 hours of cell delivery. Thirdly, the number of adult stem cells retained in the ischaemic myocardium following intravenous injection is suggested to be very small in comparsion to that accumulating in the liver, lungs, kidneys and spleen (Aicher *et al.,* 2003; Barbash *et al.,*

2003). Lastly, the initial experimental data corroborated that which had been reported by Murry *et al*., and Balsam *et al*., suggesting that donor BMMNC do not undergo differentiation into any of the cell types of myocardial lineage (Balsam *et al.,* 2004; Murry *et al.,* 2004).

Therefore, I then turned my focus to the non-regenerative aspects of adult bone marrow derived stem cell therapy. In contrast to differentiated cells, adult and embryonic stem cells highly express cytoprotective genes associated with resistance to oxidative stress, repair of DNA, and detoxifier systems (Dernbach *et al.,* 2004; Ivanova *et al.,* 2002; Ramalho-Santos *et al.,* 2002). Proteomics of stem cell also suggest that these cytoprotective genes are highly translated (Feldmann *et al.,* 2005).These essential cytoprotective mechanisms ensure bone marrow stem cell survival within their relatively hypoxic stem cell niche (Uccelli *et al.,* 2008; Wilson *et al.,* 2006). The next stage of my thesis was to elucidate the nature of BMMNC non-regenerative mechanisms and their role in attenuating acute ischaemia and reperfusion. By isolating the cardiomyocytes from the region of the myocardium subjected to ischaemia and reperfusion, I was able to show that BMMNC therapy attenuated cardiomyocyte death by reducing both cardiomyocyte apoptosis and necrosis. This attenuation of cardiomyocyte apoptosis and necrosis was most effective when BMMNC therapy was initiated at the onset of myocardial reperfusion. This observation was concordant with both the myocardial infarction data presented in chapter 3 and also with suggested homing mechanism of bone marrow stem cells i.e at the peak of the myocardial expression of SDF-1,

which is at the end of ischaemia (Askari *et al.,* 2003; Ceradini *et al.,* 2004; Jiang *et al.,* 2002; Wojakowski *et al.,* 2004).

Having suggested that BMMNC therapy afforded protection by non-regenerative means, I tested whether adult stem cell secreted factors could mediate cardioprotection *in lieu* of the cell injection. Interestingly, I found that removing the cells from BMMNC therapy did not eliminate cardioprotection. The BMMNC derived paracrine factors were, however, required to be highly concentrated such that BMS derived from at least 20 million BMMNC over at least 24 hours was necessay to replicate the cardioprotection that was afforded by 10 million BMMNC, which could be injected immediately following isolation. These data supported BMMNC mediated cardioprotection was (1) non-regenerative (2) dependent upon the trafficking of stem cells, possibly by SDF-1/CXCR4 signaling, to the ischaemic and myocardium.

In order to further understand the molecular mechanisms undelying BMMNC derived paracrine factors, I conducted a proteomic analysis of the left ventricular region that had been subjected to I/R-2h and treated with either BMMNC or BMS. The comparison of the LV proteome of hearts treated with either 10 million BMMNC or BMS, from 50 million BMMNC over 24 hours, suggested that a number of proteins might have a role in BMMNC mediated cardioprotection. Proteomic data suggested BMMNC were cardioprotective by a wide number of survival mechanims as

suggested by differential protein expression of (a) anti-apoptotic signal transduction protein 14-3-3 epsilon (b) anti-oxidants (c) heat shock proteins (d) glycolytic proteins (e) mitochondrial respiratory proteins.

The higher expression of 14-3-3 epsilon was equally up-regulated following either BMMNC or BMS therapy. An augmented expression of 14-3-3 epsilon was concordant with anti-apoptotic effects of BMMNC therapy (chapter 4) and suggested PI3K/Akt survival kinase signaling may have a major role for BMMNC mediated cardioprotection. That this was indeed the case was also suggested by the augmented phosphorylation of Akt by BMMNC therapy, as noted in the Western blots. That the downstream phosphorylation of GSK-3beta of the PI3K/Akt survival kinase signaling suggested that BMMNC therapy could have inhibited mitochondrial MPTP opening during reperfusion. Prevention of MPTP opening by BMMNC therapy was also suggested by the significantly higher expression of the mitochondrial VDAC-1.

Proteomic study of BMMNC therapy mediated cardioprotection also suggested that BMMNC therapy caused higher expression of anti-oxidants (Table 4.4.4). In connection with this, the activation of p38 MAPK during I/R is suggested to be causally linked to increased ROS, particularly H_2O_2 (Meldrum *et al.,* 1998). Activation of p38 MAPK during I/R can augment myocardial I/R injury (Chai *et al.,* 2008; Gao *et al.,* 2002; Jaswal *et al.,* 2007; Kumar *et al.,* 2004). Active p38 MAPK is suggested to augment I/R

by increasing myocardial inflammatory cytokines (Oto *et al.,* 2009; Yin *et al.,* 2008). Others, however, suggest the contrary (Steenbergen 2002). In concordance with the former, Western blotting suggested that BMMNC therapy attenuated phophorylation of p38 MAPK and also reduced the nuclear translocation of the pro-inflammatory cytokine transcription factor NF-κB.

Proteomics also eluded to other cardioprotective benefits associated with BMMNC therapy such as a higher expression of heat shock proteins, glycolytic proteins, and mitochonrial respiratory proteins. It is of course unclear as to which molecular mechanism has the principal role in BMMNC cardioprotection and future work might identify BMMNC secreted paracrine factors tp help elucidate the principal cardioprotective molecular mechanism(s).

BMMNC therapy in clincal applications where myocardial I/R is obligatory such as heart transplantation, cardiopulmonary bypass, and coronary angioplasty might be hampered by the availability of BMMNC for immediate injection upon reperfusion. Further, the injection of autologous BMMNC i.e. from patients with heart disease and other multisystem co-morbities may not provide the same therapeutic benefits demonstrated by the data presented in this thesis. Importantly, I have used freshly isolated syngeneic BMMNC, which were not be available as an "off the shelf" option for immediate injection when required. Thus, a clinically feasible

adult stem cell therapy would require a stem source that is easily available and without excessive delay. Therefore, I investigated whether the other major bone marrow stem cell type MSC might be cardioprotective in the myocardial I/R-2h rat model. The reasons for this were two fold (1) MSC are very easily isolated from whole BM and expanded in culture (2) the potential of MSC for allogeneic transplantation. These properties of MSC provides potential for MSC banking from healthy donors, for later clinical therapy in unrelated recipients. MSCs isolated from *ex vivo* whole marrow were expanded in culture and noted to be CD45 negative, CD29 positive and CD90 positive. I have shown that systemic IV injection of MSC at the onset of reperfusion could also powerfully attenuate myocardial infarction, preserve myocardial function, and prevent adverse ventricular remodeling. This suggested that an "off the shelf" BM derived stem cell for potential clinical use might be a possibility.

At this stage of my thesis, I was intrigued that the exogenous delivery of two different bone marrow derived stem cell types was powerfully cardioprotective but the endogenous bone marrow reservoir of the same stem cells types could not afford significant cardioprotection. Shintani *et al*., had, interestingly, reported an increased mobilisation of endogenous bone marrow derived stem cells following AMI (Shintani *et al.,* 2001). This was possibly due to the ischaemia augmented expression of myocardial SDF-1 and CXCR4 mediated trafficking of cells from the bone marrow niche(Askari *et al.,* 2003; Dar *et al.,* 2005; Sugiyama *et al.,* 2006).

However, despite the SDF-1/CXCR4 signaling the endogenous bone marrow stem cell mobilisation during I/R the consequential cardioprotection is insignificant compared to the cardioprotection afforded by exogenous stem cell therapy.

Initial data presented in this thesis suggested that cardioprotection by exogenous BMMNC therapy was similar to that afforded by IPC. IPC is the phenomenon attributable to the landmark study by Murry, Jennings and Reimer in 1986 and since its initial description it has become one of the most quoted scientific studies in the cardiovascular literature (Murry *et al.,* 1986). Further interpretation of this report in the context of the data presented in this thesis was interesting in a number of ways: (1) Murry *et. al.,* reported that preconditioning the heart was only cardioprotective in the short or 40 min ischaemia model but not in the 3h ischaemia model. When taken together with my data, this suggersted that the bone marrow derived stem cells could be mobilised by IPC but in the absence of reperfusion or in the absence of an SDF-1 chemokine gradient the mobilized stem cells would not be able reach the ischaemic myocardium. (2) The pattern of injury described in the 40 min ischaemia model is patchy and diffuse, which is consistent with the pattern of exogenous stem cell engraftment into the myocardium following IV injection. In contrast, the pattern of injury in the 3 h ischaemia model was solid or confluent infarcts. (3) Murry *et. al.,* also reported that pre-treatment with verapamil in the 40 min ischaemia and reperfusion model was significantly cardioprotective, but continuous

verapamil initiated 15 min after the onset of ischaemia in the 3 h ischaemia model was not cardioprotective. Following this observation, the authors suggest that verapamil pre-treatment might share a common mechanism with ischaemia pre-conditioning. Interestingly, a more recent report suggests that verapamil can disrupt the SDF-1/CXCR4 signaling axis in bone marrow derived stem cells and impair *in vitro* MSC migration (Wang *et al.,* 2008). Thus, pre-treatment with verapamil may lead to stem cell mobilization from the bone marrow; however, continuous verapamil injection could impair stem cell engraftment into the ischaemic myocardium. Indeed, blocking the CXCR4 receptor disrupts the SDF-1/CXCR4 signaling and the mobilized cells would therefore not home and engraft into tissues highly expressing SDF-1, such as the ischaemic myocardium (Liang *et al.,* 2004).

Having suggested that IPC may have a role in endogenous stem cell mobilisation led to experiments where the impact of I/R and IPC upon the endogenous BMMNC phenotype and SDF-1/CXCR4 signaling was investigated. Firstly, it was notable that I/R augmented endogenous BMMNC expression of CD45, which would be in keeping with the rise of inflammatory cytokines in the circulation following acute myocardial infarction (Lapidot *et al.,* 2002). This augmented endogenous stem cell CD45 expression suggests that the initial bone marrow response to regional myocardial I/R is to increase mobilisation and production of inflammatory leucocytes. In contrast, IPC and I/R had attenuated endogenous BMMNC

CD45 positive cells by three folds. This suggested that the preconditioned myocardium attenuated bone marrow derived inflammatory leucocytes, which is concordant with IPC mediated attenuation of myocardial acute inflammation. Moreover, endogenous BMMNC attenuated CD45 expression can impair SDF-1/CXCR4 signaling to reduce endogenous stem cell retention within the bone marrow niche (Shivtiel *et al.,* 2008). Direct disruption of CXCR4 and SDF-1 interaction, however, can lead to a massive and rapid mobilisation of endogenous bone marrow stem cells (Broxmeyer *et al.,* 2005; Sweeney *et al.,* 2002). An important endogenous mechanism that leads to the direct disruption of CXCR4 and SDF-1 interaction and de-adhesion of stem cells from their niche is the stem cell expression of CD26. CD26 is a type II transmembrane glycoprotein with extra-membranous dipeptidyl peptidase-4 activity to cleave and inactivate SDF-1 (Zhai *et al.,* 2009). In concordance with IPC having a role in increased bone marrow stem cell mobilisation, IPC augmented endogenous BMMNC expression of CD26 by six folds greater than that for the sham animals. The augmented BMMNC CD26 expression was also associated with a higher expression of CXCR4 that was approximately 10 fold higher than that for the endogenous BMMNC from sham animals. Stem cell expression of CXCR4 can be rapidly increased by externalization of intracellular stores on human CD34 positive stem cells leading to increased plasma SDF-1 mediated trafficking (Kollet *et al.,* 2003; Peled *et al.,* 1999). These findings were also in concordance with the clinical use of granulocyte colony stimulating factor

(G-CSF) for mobilization of haematopoietic stem cells by decreasing SDF-1 expression and up regulating CXCR4 expression in both human and murine bone marrow (Petit *et al.,* 2002). The magnitude of increased CD26 expression would suggest a more direct inhibition of SDF-1 within the bone marrow stem cell niche. Direct disruption of SDF-1 and CXR4 bonding by the augmented CD26 and CXCR4 expression would be consistent with a massive mobilization of endogenous bone marrow stem cells by IPC.

Hence the final experiment of the thesis whereby I demonstrated that IPC and exogenous MSC injection at the onset of reperfusion could synergistically abolish myocardial I/R injury. Further, it is also notable that the injection of 2.5 million exogenous MSC prior to the onset of 25 min regional ischaemia did not have any additional benefit to that afforded by the timely injection of MSC at the onset of reperfusion. This could be attributable due to a lack of SDF-1 expression by the myocardium prior to the onset of ischaemia and failure of the exogenous MSC to home into the myocardium. The augmented mobilization of endogenous bone marrow derived stem cells by IP might be an essential mechanism for limiting myocardial I/R injury. Further, the rapid and massive mobilization of bone marrow derived stem cells by IP might also explain the comparable myocardial protection afforded by remote ischaemic preconditioning and post-conditioning.

The data presented in this thesis is limited in the following ways: (1) there is no data to confirm that the combined effect of IPC and MSC did lead to increased exogenous and endogenous stem cell homing and engraftment within the ischaemic myocardium. (2) There is no molecular data to support the observed enhanced cardioprotection by IPC and MSC therapy. (3) The ischaemia time is short and whether the augmented protection by IP plus MSC therapy following longer ischaemia times persists is debatable.

Future experimental work should examine whether exogenous bone marrow derived stem cell therapy is also cardioprotective in a large animal model of myocardial ischaemia and reperfusion injury e.g. the porcine model. Subject to large animal data, this method of cardioprotection might be translatable into clinically where myocardial ischaemia and reperfusion is obligatory such as heart transplantation, cardiopulmonary bypass and coronary angioplasty.

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